Studies on Genomic Alterations in HER2-Positive Breast Cancer–Focus on Design, Synthesis & Evaluation of Anilinoquinazoline Analogues as Potential HER2 inhibitors

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By

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CERTIFICATE

I declare that the thesis entitled "**Studies on Genomic Alterations in HER2-Positive Breast Cancer–Focus on Design, Synthesis & Evaluation of Anilinoquinazoline Analogues as Potential HER2 inhibitors**" has been prepared by me under the guidance of Prof. Anjana Munshi, Head of the Department, Human Genetics and Molecular Medicine and Dr. Vinod Kumar, Assistant Professor, Department of Pharmaceutical Sciences and Natural Products, School of Basic and Applied Sciences, Central University of Punjab, Bathinda. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

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Keywords: HER2-positive breast cancer, overexpression/amplification, trastuzumab resistance, anilinoquinazoline, MTT assay, antiproliferative activity

Human epidermal growth factor receptor 2-positive (HER2-positive) breast cancer is an aggressive breast cancer subtype characterized by HER2 overexpression/amplification. Genomic alterations of HER2 and others have been reported to be associated with, HER2 overexpression and prediction of trastuzumab-response. The current study was carried out to identify genomic alterations associated with HER2-positive breast cancer and evaluate their association with clinical outcome in response to trastuzumab therapy given to HER2-positive breast cancer patients. Global Sequencing Array (GSA) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques were used to determine alterations in HER2 and other HER2-interacting as well as signaling-related genes implicated in the disease. In addition, 20 formalin fixed paraffin-embedded (FFPE) tissue samples were also evaluated by GSA for identifying significant variations associated with the disease as well as response to trastuzumab therapy. A germline variant in HER2 gene (I655V) was found to be significantly associated with the risk of the disease ($p < 0.01$). A nonsense mutation in PTPN11 (K99X), a pathogenic CCND1 splice site variant (P241P), a hotspot missense mutation in PIK3CA (E542K) and a hotspot missense mutation in TP53 (R249S); were observed in 25%, 75%, 30% and 40% of the HER2-positive breast cancer tissue samples, respectively. Mutant CCND1 (P241P) and PIK3CA (E542K) were found to be significantly associated with reduced disease-free survival (DFS) in patients treated with trastuzumab (p: 0.018 and 0.005, respectively). These results indicate that HER2, PTPN11, CCND1 and PIK3CA genes are important biomarkers in HER2-positive breast cancer. Moreover, the patients harboring mutant CCND1 and PIK3CA exhibit a poorer clinical outcome as compared to those carrying wild-type CCND1 and PIK3CA.

Development of resistance and disease-relapse are the major problems associated with trastuzumab. Tyrosine-kinase inhibitors (TKIs) present better option to address the issues associated with trastuzumab. However, problems of resistance and ineffectiveness as monotherapy; persist with the currently available TKIs as well. We synthesized anilinoquinazoline-based compounds and evaluated them for anti-proliferative activity against HER2-positive breast cancer. Of the synthesized compounds (HS-2, HS-3, HS-5, HS-8 and HS-9), three (HS-3, HS-5 and HS-8) were evaluated for biological activity. HS-8 proved to be most-effective against SKBR3 (HER2-positive breast cancer cells) (IC_{50}=2.8μM)

with a lesser cytotoxicity towards the MDA-MB-231 (Triple-negative breast cancer cells) (IC_{50}=3.2µM) and no toxicity towards FR-2 (normal breast epithelial cells).

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I want to dedicate my thesis to my mother, **Mrs. Darshana Singla** and elder sister, **Nainy** for their blessings, guidance and confidence in my abilities. Last, but not least I want to thank almighty **God** for granting me strength to move forward.

Heena Singla

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LIST OF ABBREVIATIONS

Abbreviation	Full Form
ADP	Adenosine Diphosphate
ADRs	Adverse Drug Reactions
ATP	Adenosine Triphosphate
ASCO-CAP	American Society of Clinical Oncology/College of American Pathologists
BL	Basal Like
BRAC1	Breast Cancer Type 1
Cdx-VDR2	Caudal Type Homeobox 2 Vitamin D Receptor
CCR2	Chemokine Coreceptor 2
CEP17	Chromosome Enumeration Probe 17
CCND1	Cyclin D1
CDK4	Cyclin Dependent Kinase 4
CYP	Cytochrome P450
DHFW	Department of Family and Health Welfare
DMSO	Dimethy Sulfoxide
DFS	Disease Free Survival
dd	Doublet of doublet
DCIS	Ductal Carcinoma *In Situ*
DMEM	Dulbecco's Modified Eagle's medium
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ESR1	Estrogen Receptor 1
FBS	Fetal Bovine Serum
FISH	Fluorescence In Situ Hybridization
GSA	Global Screening Array
Hsp	Heat Shock Protein
Hz	Hertz
HDAC	Histone Deacetylase
HR	Hormone Receptor
HRT	Hormone Replacement Therapy
HER2	Human Epidermal Growth Factor Receptor 2
IHC	Immunohistochemistry
IAP	Inhibitor of Apoptosis
IGF-1 R	Insulin Like Growth Factor 1 Receptor
IDC	Invasive Ductal Carcinoma
ILC	Invasive Lobular Carcinoma
KRAS	Kirsten Rat Sarcoma
LCIS	Lobular Carcinoma *In Situ*
MACC1	Metastasis Associated Colon Cancer Protein 1
µM	Micromolar
MAPK	Mitogen Activated Protein Kinase 1
MUC	Mucin
nm	Nanomolar

ppm	Parts Per Million
PTEN	Phosphatase and Tensin Homolog
PBS	Phosphate Buffer Saline
PI3CKA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PI3K	Phosphoinositide 3-Kinase
PLC-y	Phospholipase C gamma
PCR	Polymerase Chain Reaction
PR	Progesterone Receptor
PKB/Akt	Protein Kinase B
PTP	Protein Tyrosine Phosphatase
PTPN11	Protein Tyrosine Phosphatase, Non-Receptor Type 11
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
RBC	Red Blood Cells
RFLP	Restriction Fragment Length Polymorphism
SNP	Single Nucleotide Polymorphism
S.D.	Standard Deviation
SAR	Structure Activity Relationship
SULT1A1	Sulfotransferase Family 1A Member 1
TDLU	Terminal Ductal Lobular Unit
TLC	Thin Layer Chromatography
TOP2A	Topoisomerase 2 A
TN	Triple Negative
TP53	Tumor Suppressor Protein 53
TKI	Tyrosine Kinase Inhibitor
TK	Tyrosine Kinase
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Cancer is a global health hazard claiming millions of lives, each year. It refers to the unimpeded growth of some abnormal cells of the body. These expeditiously growing cells have tendency of invasion into nearby tissues as well as the distant parts of the body. The word cancer originated from Greek term “carcinoma” first used by Hippocrates (460-370 B.C.) for ulcer-forming tumors. Carcinoma refers to crab, the Latin word for which is cancer (Skuse, 2015). Cancer arising in the breast tissue is referred to as the “breast cancer”. Among the various malignancies, breast cancer is the most common malignancy found in the female population (Bray *et al.*, 2018). However, male breast cancer cases have also been reported, although at a much lower frequency in comparison with female breast cancer (Siegel *et al.*, 2015, 2017). For both the sexes combined, female breast cancer is the second most common cause of cancer deaths after lung cancer. The disease accounts for 11.6% of the total number of deaths due to cancer (Bray *et al.*, 2018). In developing countries like India, the prevalence of this dreadful disorder is quite high with approximately 10,000 new breast cancer incidences per year (Goyal *et al.*, 2015; Gupta, 2016; Katkuri *et al.*, 2018). Alarmingly high breast cancer prevalence was recorded in Punjab, especially in its Malwa region. As per the survey conducted by Department of Health and Family Welfare (DHFW) in 2013, the prevalence of this disease in Malwa was higher than rest of the state and even higher than the national average (Aggarwal *et al.*, 2015). Notwithstanding, some recent findings of experts based on the cancer registry of Punjab pointed out that the cancer rate in Malwa is not that disturbing (www.timesofindia.indiatimes.com).

Breast cancer can’t be termed as a single disease. Heterogeneous disease is a better phrase for this dreaded syndrome. Variable clinical treatment and differential response towards treatment for various subtypes of breast cancer reflects the heterogeneity of this disease (Viale, 2012). Depending upon the histopathology, prediction analysis of microarray 50 (PAM50) gene expression, expression of immuno-histochemical markers and various other factors breast cancer has been classified, accordingly.

Histopathological classification (based on microscopic examination) and molecular classification (based on immuno-histochemical markers) are the major classifications of breast cancer. Histopathologically, it is generally divided into 2 types: carcinoma *in situ* and invasive carcinoma as per the invasiveness of the tumors. *In situ* breast carcinomas are non-invasive but potentially malignant, whereas invasive breast cancers refer to malignant abnormal proliferation of neoplastic cells in the breast tissue. Based on the site from which the tumor originated, invasive carcinoma and carcinoma *in situ* are further classified as ductal (originating from ducts) and lobular (originating from the lobules) subtypes (Dunnwald *et al.*, 2007; Vinay *et al.*, 2010; Munagala *et al.*, 2011; Makki, 2015)**.** Based on the expression of immuno-histochemical markers, breast cancer is classified into 3 major types: Hormone receptor (HR)-positive, Human epidermal growth factor receptor 2 (HER2)-positive and Triple-negative (TN) breast cancers (Giuliano *et al.*, 2013). HR-positive breast cancers express hormone receptors *i.e.,* Estrogen receptor (ER) and/or Progesterone receptor (PR), whereas HER2-positive breast cancers express HER2 and/or ER/PR. In contrast, TN breast cancers fail to express all the three receptors (ER, PR and HER2) (Giuliano *et al.*, 2013).

HER2-positive breast cancer is a rare but distinct subtype that tends to grow with more aggression compared to other breast cancer subtypes. It comprises 15-30% of overall breast cancer cases and is generally characterized by high histologic grade, chances of increased metastasis and reduced survival (Morrow *et al.*, 2009; Ross *et al.*, 2009; Gutierrez *et al.*, 2011). HER2 tyrosine kinase (TK) receptor is a member of Epidermal growth factor receptor (EGFR) family and is encoded by HER2/ErBb2/neu2 gene occupying human chromosomal locus 17q12-21.32. HER2-positive status denotes overexpression of HER2 receptor and/or HER2 gene amplification. HER2 overexpression/amplification causes homodimerization of HER2 receptors and enhanced heterodimerization of HER2 with its other EGFR family members *viz.,* HER1, HER3 and HER4 (Neve *et al.,* 2001; Iqbal *et al.,* 2014). HER2 possesses strongest catalytic kinase activity and upon heterodimerization it confers vigorous signaling activity. It is the favourable

dimerization partner for the other constituents of EGFR family. HER2 forms most-potent heterodimer with kinase-inactive HER3, stimulating the anti-apoptotic Phosphoinositide 3-kinase (PI3K) pathway. HER2-mediated cell signaling can also be induced upon interaction with insulin-like growth factor receptor-1 (IGFR-1) and ER (Gutierrez & Schiff, 2011a). As a result of homo-/heterodimerization, multiple downstream cell-signaling pathways such as PI3K pathway, Mitogen-activated protein kinase (MAPK) pathway, Phospholipase C-γ (PLCγ) and other cell-signaling pathways are activated. This ultimately causes enhanced cell proliferation and delayed apoptosis, hence, contributing towards HER2-positive carcinogenesis (Tai, Mahato, & Cheng, 2010; Iqbal *et al.*, 2014).

The exact cause of breast cancer including HER2-positive breast cancer is not known (www.moffitt.org) (Singla *et al.*, 2017). However, there are certain factors known to increase an individual's risk of developing breast cancer. Factors such as age, sex (being female), age at menarche, age at menopause, first pregnancy after age of 30 years, family history of breast cancer and genetic aberrations are the non-modifiable risk factors. On the other hand, factors like hormone replacement therapy (HRT), being alcoholic, obesity, smoking and exposure to radiations are the modifiable risk factors (Sharma *et al.*, 2010; Canaria *et al.*, 2014; Maas *et al.*, 2016).

Genomic alterations are significant biomarkers that predict the risk of HER2-positive breast cancer (Singla *et al.*, 2017). Variants of HER2, as well as of other genes including Cytochrome-P450 genes (CYP19A1, CYP1A1*2, CYP17), Estrogen receptor-1 (ESR1), Kirsten rat sarcoma (KRAS), Vascular endothelial growth factor A (VEGFA), breast cancer type-1 (BRCA1), Metastasis-associated in colon cancer protein 1 (MACC1), Sulfotransferase family 1A member 1 (SULT1A1), Caudal type homeo box 2 vitamin-D receptor (Cdx2 VDR), and Chemokine coreceptor-2 (CCR2) have been reported to be associated particularly with HER2-positive breast cancer susceptibility (Singla *et al.*, 2017). Genomic alterations might not have direct influence on HER2 overexpression. But, these may interfere with the intracellular pathways involved in HER2 signaling, thus, aggravating the process of HER2-positive breast carcinogenesis.

For the treatment of HER2-positive breast cancer, monoclonal antibodies and tyrosine-kinase inhibitors (TKIs) represent two key classes of drugs. Antibody-drug conjugates represent another class of HER2 targeted inhibitors (Moasser *et al.*, 2015). Trastuzumab, pertuzumab (monoclonal antibodies); trastuzumab-biosimilars (trastuzumab-dkst, trastuzumab-pkrb and trastuzumab/hyaluronidase-oysk); ado-trastuzumab emtansine (antibody-drug conjugate); lapatinib and neratinib (HER2 TKIs); are the approved drugs for the treatment of HER2-positive breast cancer. However, trastuzumab is the most-commonly employed and efficient first–line drug for the treatment of HER2-positive breast cancer (www.fda.gov). But the development of resistance, thereby; refractory disease is the major limitation in case of trastuzumab (Hurvitz *et al.*, 2013). Alterations in the genes involved in HER2 signal transduction pathways mainly PI3K pathway such as Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mutations, Phosphatase and tensin homolog (PTEN) loss and Protein-kinase B (PKB, known as Akt) mutations, Fc-gamma receptor (FcgR) polymorphisms and others are reported to be associated with resistance towards trastuzumab-therapy (Singla *et al.*, 2017). Cardiac toxicity, the adverse event associated with trastuzumab therapy is another limitation.

To avoid the drawbacks of trastuzumab-therapy; antibody-drug conjugate, ado-trastuzumab emtansine was also developed. But, in this case also problem of adverse drug reactions (ADRs) persists. There are various mechanisms that cause resistance towards antibody treatment. However, formation of the spliced HER2 protein (p95HER2) is the major mechanism of resistance towards antibody-based treatment. p95HER2 lacks the extracellular domain where the antibody binds, but remain kinase-active. Hence, trastuzumab-resistant HER2 can be successfully targeted by HER2 TKIs. HER2 TKIs are the small-molecules that compete for Adenosine-triphosphate (ATP) binding site of the intracellular catalytic domain of HER2 kinase, thus, inhibiting protein phosphorylation, a key event for initiation of signal transduction (Moasser *et al.*, 2015). The approved TKIs (lapatinib and neratinib) are orally bioavailable, as opposite to the intravenous administration of antibodies and possess reduced risk of cardiac toxicity.

Lapatinib effectively inhibits trastuzumab-resistant truncated HER2 protein, p95HER2 (Xia *et al.*, 2004). It also has been reported to induce apoptotic effects in trastuzumab-resistant cell lines (Nahta *et al.*, 2007).

Another merit of lapatinib is its tendency to cross blood-brain-barrier (BBB) contradictory to the monoclonal antibodies which are unable to cross BBB. Hence, small-molecules may prove effective treatment for curing central nervous system metastasis in HER2-overexpressing breast cancer patients (Lin *et al.*, 2008). However, there are reports of development of resistance against lapatinib and neratinib (Xia *et al.*, 2006; Sergina *et al.*, 2007; Breslin *et al.*, 2017). Hence, novel drug candidates are required to address various issues with the existing therapeutics. In recent years several new molecules emerged as potent leads as TKIs to target HER2 domain. Some of these molecules are in advance stage of clinical trials (clinicaltrials.gov). Hence, still there is requirement of more efficacious drug candidates in order to overcome the resistance developed against the approved therapies and to enhance the treatment selectivity. Hence, a number of novel molecules are being investigated as potent TKIs. Important leads are being identified through various structure-activity relationship (SAR) studies possessing higher kinase-selectivity and improved pharmacokinetic profile. Although the specificity of TKIs is less than that of monoclonal antibodies, but the reduced toxic events and other potential advantages, surpass the unspecific behavior of TKIs (Singla *et al.*, 2018).

The current study was designed with an aim of screening genomic alterations in HER2-positive breast cancer in Malwa region of Punjab and evaluation of these alterations with HER2-positive breast cancer risk and trastuzumab resistance. In addition, we aimed to synthesize anilinoquinazoline-based derivatives as putative HER2 inhibitors. The proposed synthetic analogues might prove useful in overcoming the limitations of trastuzumab such as resistance, ADRs and inability to cross BBB. Further, the genomic alterations associated with trastuzumab resistance reported earlier or found in the present study might not confer resistance towards the proposed molecules. The objectives of the study are:

1.1. Evaluation of demographic profile of the HER2-positive breast cancer in the Malwa region of Punjab.

1.2. Evaluation of association of HER2 and other gene variants with HER2-positive breast cancer susceptibility.

1.3. Evaluation of association of HER2 and other gene variants with clinical outcome in response to trastuzumab-therapy given to HER2-positive breast cancer patients.

1.4. Design, synthesis and biological screening of anilinoquinazoline analogues against HER2-positive breast cancer.

CHAPTER 2
REVIEW OF LITERATURE

Breast cancer refers to the malignant disease affecting the cells of mammary gland and is a common cancer largely affecting the female. The disease is 100 times more common in females than in males and may arise either in milk-producing lobules or in the ducts those connect the lobules to the nipple. In case of small tumor, no symptom other than a painless lump is observed. Skin irritation or dimpling, pain, nipple retraction, redness, scaliness, redness, changes in the appearance of breast skin or nipple discharge are some other symptoms seen among breast cancer patients. Breast cancer is a group of diseases but not a single disease characterized by different risk factors, subtypes, clinical features and response to therapeutic strategies (DeSantis *et al.*, 2013).

2.1. Breast cancer epidemiology

Breast cancer is the most commonly diagnosed cancer in females. It occupies nearly a quarter of all cancers in women. For both the sexes combined, it is the second most common cancer after lung cancer (Bray *et al*., 2018). About 1.7 million women were diagnosed with breast cancer and 522,000 deaths occurred due to breast cancer, as per 2012 GLOBOCAN statistics. Thus, there was an increase of nearly 18% from 2008 to 2012 in breast cancer incidence and related mortality (Tao *et al.*, 2015). Breast cancer is a global problem and is affecting the developing countries in growing proportions. The burden of this disease is expected to cross 2 million by 2030, globally (Gupta *et al.*, 2015). By 2050, the breast cancer incidences are expected to rise upto 3.2 million new cases per year, worldwide (Tao *et al.*, 2015). According to WHO, more than 2 million new cases of breast cancer were reported in 2018.

In 2018, GLOBOCAN estimated the incidence and mortality for 36 cancers in 185 countries. In the vast majority of the countries (154 of 185) breast cancer has been reported to be the most frequently diagnosed cancer. In over 100 countries, it is also the major cause of cancer death with major exceptions of Australia/New Zealand, Northern Europe, Northern America and many countries in Sub-Saharan Africa. In Australia/New Zealand, Northern Europe, Western Europe, Southern Europe, and Northern America; the breast cancer incidence

rates are the highest. The mortality rates on account of breast cancer are the highest in Melanesia (Fiji), worldwide (Bray *et al.*, 2018).

2.2. Prevalence in India

In India, the incidences and mortality rates as a result of breast malignancy are continuously increasing (Gupta *et al.*, 2015). According to GLOBOCAN (WHO), for year 2012, India recorded 70, 218 mortalities due to breast cancer which was quite high in comparison with any other country in the world (Bodh *et al.*, 2015). Every year, about, 10,000 breast cancer incidences occur in India (Goyal *et al.*, 2015).

According to some previous reports, the prevalence of breast cancer in Malwa belt of Punjab is the highest in the country and the incidences of the disease are increasing at alarming frequency. As per a door-to-door survey conducted by DHFW in 2013, a higher cancer prevalence in Malwa (1089/million/ year) was reported in comparison with other two regions of the state i.e. Majha (647/million/year) and Doaba (881/million/year) (Bedi *et al.*, 2013). For a long time, Malwa region of Punjab specifically the cotton growing area has been viewed as a focal point of cancer cases in Punjab (Oberoi *et al*., Nanda *et al*.). However, some most recent reports published in year 2017 acclaimed that cancer incidences are even higher in the non-cotton growing regions including Amritsar and Ludhiana leaving behind the cotton sowing areas of Malwa. Amritsar (Majha) (4,692 cases) was enlisted at the top and then, Ludhiana (Doaba) (4,052 cases) followed by Bathinda (Malwa) (3,250 cases), Gurdaspur (Majha) (2,859 cases), Jalandhar (Doaba) (2,801 cases) and Tarn Taran (Majha) (2,204 cases) in a subsequent order. This data was based on the number of cancer cases approved for financial help across Punjab from January 2012 to August 2017, by the Punjab Health Department (timesofindia.indiatimes.com). Based upon the cancer registries maintained at Sangrur, Mansa, Ajitgarh and Chandigarh; experts of Tata Memorial Centre concluded that the rate of cancer incidence in Punjab is comparable to the national average and is not that alarming. In fact, rural areas recorded a predominantly lesser number of cases than urban areas. In addition, oesophageal cancer was found to be the most prevalent followed by breast cancer and, then cervical cancer (www.tribuneindia.com).

2.3. Breast cancer classification

Breast cancer reflects a high degree of heterogeneity in terms of genetic as well as clinical aspects. This necessitated the requirement of a scientifically sound, clinically relevant and widely reproducible classification of this heterogeneous disorder. Traditionally, breast cancer has been classified into several histopathological types based upon tumor origin, aggression, staging, grading, microscopic features and other morphological characteristics. In the due course of time and with the advancement of molecular techniques such as gene-expression profiling (GEP) and transcriptomics led to the establishment of molecular markers. Transitioning through the ages, molecular classification of breast cancer came into the picture that's based upon the molecular markers mainly immunohistochemistry (IHC) surrogates (Viale, 2012; Eliyatkın *et al.*, 2015). The histopathological and molecular classification systems have been discussed in detail.

2.3.1. Histopathological classification

The microscopic features of the breast tissue are taken into consideration while classifying breast cancers, histopathologically. Breast cancers are broadly categorized into 2 major groups: *in situ* and invasive breast cancers depending upon the pathological characteristics and invasion-properties. As per the origin of tumor, these are further divided into various subtypes (Fig. 1) (Makki, 2015).

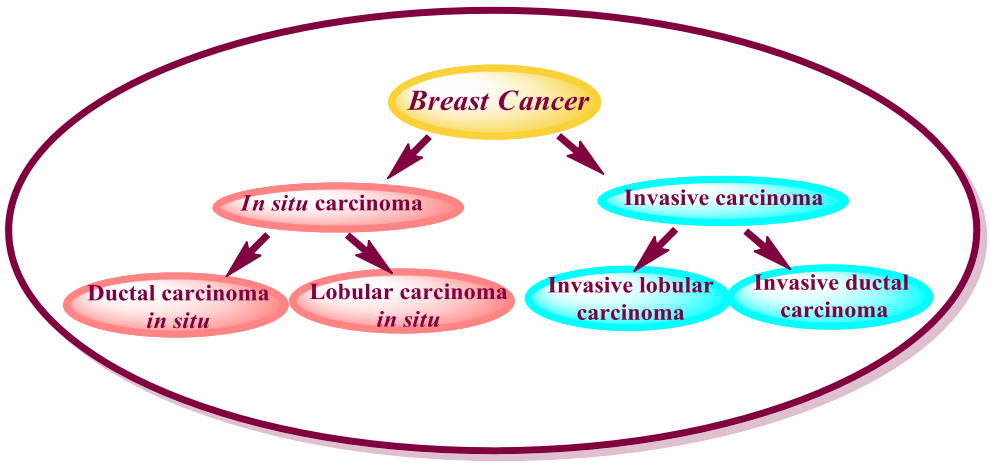


Fig. 1: Histopathological classification of breast cancer

2.3.1.1. *In situ* (non-invasive) breast cancer

In situ breast cancer is divided into two subtypes: lobular carcinoma *in* situ (LCIS) and ductal carcinoma *in situ* (DCIS).

i. **Lobular carcinoma *in situ* (LCIS):** It is the intralobular proliferation of small, fairly uniform, and loosely cohesive cells, originating in the terminal ductal lobular unit (TDLU), with or without pagetoid involvement of terminal ducts. Microscopically, LCIS generally leaves the underlying architecture intact and is recognizable as lobules. It has no distinguishing features on gross examination and is usually found incidentally in breast specimen or biopsy performed for other reasons. It is multicentric in about 70% of cases and bilateral in approximately 30%–40% of cases (Makki, 2015).

ii. **Ductal carcinoma in situ (DCIS):** It is a non-invasive breast cancer which develops within the pre-existing normal ducts and is also referred to as intraductal carcinoma. Though non-invasive, these cancers have high potential to become invasive cancers in a due course of time (Feng *et al.*, 2018).

2.3.1.2. Invasive or infiltrating breast cancer

Invasive breast cancers are characterized by the invasion, hence, escalation of cancer cells outwards breast lobules and ducts and growth into the surrounding breast stromal tissue. On a whole, about 90-95% of all breast cancer cases fall in the subcategories of invasive breast cancer (Feng *et al.*, 2018). Invasive breast cancers are further divided into following two types, based on the tissue and cell types involved:

i. **Invasive ductal carcinoma (IDC):** It is the most common type of breast cancer that comprises nearly 80% of all breast cancers (Feng *et al.*, 2018).

ii. **Invasive lobular carcinoma (ILC):** It is the second most common type of breast cancer. Approximately 10-15% of all breast cancers are ILCs (Feng *et al.*, 2018).

WHO 2013, classification of breast tumors covers not only invasive breast cancers, but also precursor lesions, lesions of low malignant potential, benign epithelial proliferations, fibroepithelial, myoepithelial and mesenchymal neoplasms, among others (Sinn *et al.*, 2013). Even, benign (non-cancerous) as well as malignant (cancerous) and some very rare forms of breast tumors including male breast tumors have been included in this classification, making it

much complex (Sinn *et al.*, 2013). The traditional histopathological classification is adopted worldwide and is reasonably reproducible. This classification actually doesn't mirror the much wider heterogeneity of breast cancer, as it groups the tumors having a very different biological and clinical profile together within the same class. Hence, a classification that is more relevant clinically as well as biologically was required such that suitable therapeutic strategies could be tailored, accordingly.

2.3.2. Molecular classification of breast cancer

Based on the expression of established immunohistochemical (IHC) biomarkers, such as ER, PR, HER2 and Ki-67 estrogen receptor breast cancer is broadly classified as follows:

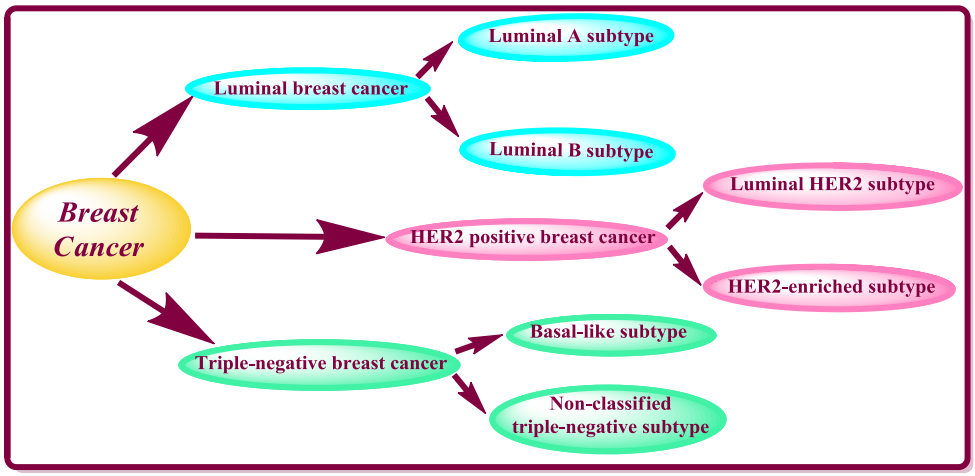


Fig. 2: Molecular classification of breast cancer

2.3.2.1. ER+/ luminal tumors: Divided into two subtypes:

i. **Luminal A subtype:** About 30-40% of invasive breast cancers belong to luminal A subtype. Luminal A tumors are often characterized by an IHC profile with high ER and PR expression, no HER2 expression, and low Ki-67. Luminal A tumors bear PIK3CA mutation in higher frequency, whereas tumor-suppressor protein 53 (p53)-mutation in lower frequency (Tang *et al.*, 2016).

ii. **Luminal B (luminal B HER2-negative) subtype:** Luminal B subtype comprises 20-30% of all invasive breast cancers. A low to moderate expression of the luminal-specific genes, including the ER cluster, but not HER2 are some specific features of this subtype. These cancers exhibit a lower frequency of PIK3CA mutations and a higher frequency of p53 mutations, when compared

with luminal A cancers. These cancers are distinguished by a lower level of ER expression, a lower level of or negative PR expression, and a higher level of Ki-67 labeling (Tang *et al.*, 2016) (Fig. 2).

2.3.2.2. HER2-positive breast cancer

HER2 positive breast cancers exhibit HER2 overexpression and/or amplification. This subgroup consists of 15% to 20% of all invasive breast cancers. HER2-positive tumors are very heterogeneous at the molecular level. HER2-positive tumors should be divided into two subtypes: luminal HER2 subtype (ER and/or PR positive/HER2-positive) and HER2-enriched subtype (ER and/or PR-negative/HER2-positive) (Tang *et al.*, 2016).

i. **Luminal HER2 (luminal B, HER2-positive) subtype:** About half of HER2-positive invasive breast cancers fall into this luminal HER2 subtype. They are ER positive, although often at lower levels compared with HER2-negative tumors (Tang *et al.*, 2016).

ii. **HER2-enriched Subtype:** This subtype is characterized by low to absent gene expression of ER and several additional transcriptional factors expressed in the luminal/ER-positive cluster. It has high expression of several genes in the HER2 gene amplicon at 17q22.24, including HER2 and growth factor receptor bound protein 7 (GRB7). About 71% of the tumors in this subtype have a p53 mutation and activation of receptor tyrosine kinase pathways, such as fibroblast growth factor receptor 4 (FGFR4), HER1, and HER2; and 39% of tumors have a PIK3CA mutation (Tang *et al.*, 2016) (Fig. 2).

2.3.2.3. Triple-Negative Breast Cancer

Triple-negative breast cancer is defined by the lack of expression for ER, PR, and HER2. It has two subtypes: basal-like (BL) and non-classified TN subtype.

i. **Basal-like (BL) subtype:** About 15% of all invasive breast cancers belong to this subtype, and there is a great diversity in the histologic features, mutation profiles, response to chemotherapy, metastatic behavior, and survival. Breast cancers of BL subtype show high expression of many of the genes characteristic of breast basal epithelial cells, such as keratins 5 and 17, laminin, and fatty acid–binding protein. There have been p53 mutations reported in 82% of these BL breast cancers, and most are positive for keratins 5/6 and 17 (Tang *et al.*, 2016).

ii. **Non-classified TN subtype:** There is a small group of TN breast cancers that are also negative for CK5 and EGFR. This subgroup of tumors possesses better breast cancer–specific survival and disease-free survival (DFS) compared with BL subtype and are less likely to be associated with a BRCA1 mutation (Tang *et al.*, 2016) (Fig. 2).

2.4. HER2-positive breast cancer: Rare but distinct

HER2-positive breast cancer is rare, constituting only 15–30% of all breast cancers (Iqbal *et al.*, 2014; Loibl, 2017). The incidence rates of HER2 positive breast cancer may vary across different ethnic groups. Korean, Filipina, Chinese, and Southeast Asian women belonging to Asian-Pacific Islander (API) group of the U.S. were reported to have a higher risk of HR-negative/HER2-positive breast cancers compared with Non-Hispanic black (NHB) women of U.S.; whereas Japanese (API sub-ethnic group) and American-Indian (AI) women were at a lower risk for this subtype. However, the incidence rate of HR-negative/HER2-positive breast cancer for the API women as a whole was similar to rates among other ethnic groups of the U.S. including American Indian/Alaska Native (AI/AN), API, NHB and non-Hispanic white (NHW) women (DeSantis *et al.*, 2017).

HER2 positive breast cancer represents an aggressive phenotype and is generally of high histologic grade (Ross *et al.*, 2009; Arpino *et al.*, 2015). Compared to other types of breast carcinomas, HER2-positive breast carcinomas possess distinct biological features such as increased potential of metastization to brain and viscera (Gutierrez *et al.*, 2011; Arpino *et al.*, 2015). A significantly reduced DFS and overall survival is seen in HER2 positive breast cancer patients (Morrow *et al.*, 2009; Sirkisoon *et al.*, 2016).

2.5. HER2 tyrosine kinase protein

HER2 (also known as ErBb2/c-ErBb2/neu), is a 1255 amino acid, 185 kDa tyrosine kinase receptor protein (p185), encoded by HER2/ErBb2/neu gene located on the long arm of chromosome 17q (17q12) (Brennan *et al.*, 2000; Park *et al.*, 2008; Tai *et al.*, 2010; Iqbal *et al.*, 2014; Sasaki *et al.*, 2015). The discovery of the HER2 oncogene was first made in 1984 by Weinberg and associates at Massachusetts Institute of Technology, Rockefeller, and Harvard University (Schechter *et al.*, 1984). HER2 belongs to the class-I receptor tyrosine

kinase (RTK) family of transmembrane glycoproteins i.e. EGFR family, comprising HER1 (EGFR/ErBb1), HER2 (ErBb2, neu), HER3 (ErBb3) and HER4 (ErBb4), all cherishing tyrosine phosphorylation activity upon dimerization (Park *et al.*, 2008; Zhou *et al.*, 2013). Three functional domains i.e. an extracellular domain for ligand binding, a transmembrane domain, and an intracellular tyrosine kinase domain, are possessed by all the EGFR family members (Damodaran *et al.*, 2012). HER2 is unique among the other EGFR family members, as it is not dependent on ligand for activation (Roskoski, 2004; Damodaran *et al.*, 2012). It undergoes homodimerization only when there is overexpression of HER2 protein. Heterodimerization of HER2 with other members of EGFR family particularly the kinase-inactive HER3, results into most potent signaling activity (Iqbal *et al.*, 2014).

2.6. Functional significance of HER2

HER2 is crucial for the normal developmental processes in humans (Park *et al.*, 2008). It plays a significant in cell signal transduction pathway and hence, regulates cell proliferation, differentiation, survival, and migration. It is required for embryonic development and tissue maintenance in adults. Multiple organs express HER2 and activation of HER2, is required for various physiological processes, i.e., oligodendrocyte formation, Schwann cell myelination and radial glia establishment in cerebral cortex during nervous system development; cardiogenesis; the adult cardiac function; cardiomyopathy prevention in adult heart; normal mammary tissue development and muscle spindle maintenance (Eccles, 2011; Brix *et al.*, 2014).

2.7. HER2 overexpression/amplification

Overexpression of HER-2 protein was reported first by Van *et al.* in 1988 in *in situ* breast carcinoma (Van *et al.*, 1988). HER2 gene amplification and protein overexpression are associated, as there are rare incidences of single copy overexpression. In 90% of the HER2-positive breast cancers, the copy number gain involving HER2 locus i.e. gene amplification is seen. However, it is not necessary that HER2-overexpression and HER2 amplification will always accompany each other. Tumors deficient in HER2 amplification may show protein overexpression and tumors deficient in protein overexpression may show HER2 amplification, suggesting other alternative mechanisms for HER2

overexpression (Puputti *et al.*, 2006). Polysomy 17 (presence of extra copies of chromosome 17) is an alternative mechanism responsible for the HER2 overexpression (Hanna *et al.*, 2014). Amplification/overexpression of HER2 gene is found to be associated with 15-30 % of the breast cancer patients (Iqbal *et al.*, 2014). There can be up to 25-50 copies of HER2 gene and a 40-100 fold increase in HER2-protein in breast carcinomas, hence, resulting in the expression of about 2 million receptors at the tumor cell surface (Gutierrez *et al.*, 2011).

HER2 overexpression has been linked to the increased formation of both homodimers and heterodimers (Moasser, 2007), which leads to increased cell signaling and, in turn, results in the increased cell proliferation, cell motility, tumor invasiveness, progressive regional and distant metastases, accelerated angiogenesis and reduced apoptosis (Ross *et al.*, 2009). Amplification/overexpression of HER2 gene is found to be associated with 15-30 % of the breast cancer patients (Iqbal *et al.*, 2014). There can be up to 25-50 copies of HER2 gene and a 40-100 fold increase in HER2 protein in breast carcinomas, hence, resulting in the expression of about 2 million receptors at the tumor cell surface (Gutierrez *et al.*, 2011).

IHC and fluorescence *in situ* hybridization (FISH) are the two major techniques used for determining HER2 status. IHC evaluates HER2 overexpression, while FISH determines about HER2 gene amplification. According to the American Society of Clinical Oncology/College of American Pathologists (ASCO-CAP), an IHC score of 3+, with strong staining of more than 10% of all invasive tumor cells accounts for HER2-positive status. IHC scores of 0 and 1+ are indicative of HER2 negative status, whereas an IHC score of 2+ needs further confirmation by FISH. A single-probe FISH assay determines the HER2 gene copy number and a dual-probe FISH evaluates the ratio between the HER2 gene copy number and the chromosome enumeration probe 17 (CEP17). An average HER2 copy number of ≥ 6.0 signals/cell or a HER2/CEP17 ratio of ≥ 2.0 or defines the presence of HER2 amplification (Schramm *et al.*, 2015; Loibl *et al.*, 2017).

2.8. HER2 signaling

As HER2 has no direct activating ligand, it may exist in constitutively active form as a homodimer or may be activated upon heterodimerization with the other members of the EGFR family. As a consequence of homodimerization or heterodimerization of HER2, the autophosphorylation of tyrosine kinase residues result, hence, activating a plethora of signaling pathways i.e.PI3K, MAPK, PLCγ, and other pathways (Tai *et al.*, 2010; Iqbal *et al.*, 2014), which, in turn, regulate cell growth, survival and differentiation (Arteaga *et al.*, 2012) (Fig. 3).

PI3K and MAPK are the major pathways triggered by the dimerization of RTKs. Upon dimerization, tyrosine kinases are activated inducing C-terminal tyrosine phosphorylation. The cytoplasmic domain HER2 possesses numerous phosphorylation sites those are essential for protein-protein interactions and signaling cascade-induction. Tyr1139, Tyr1222 and Tyr1248 involved in the activation of the RAS/MAPK pathway along with Tyr1196 needed for initiation of the PI3K/AKT pathway are the most commonly studied phosphorylation sites (Dittrich *et al.*, 2014).

2.8.1. The PI3K pathway

The major pathway that is activated by HER2 is the PI3K signaling pathway which has itself become a target for therapy in cancer, due to its role in cell growth, survival, proliferation as well as protein synthesis, invasive properties and drug resistance. PI3K/AKT pathway is induced by dimerization of the two RTKs and the subsequent phosphorylation of the C-terminal domain. Activation of PI3K/Akt that is regulated by phosphatase and tensin homolog (PTEN) leads to the upregulation of various antiapoptotic proteins resulting in delayed p53-mediated apoptosis (Park *et al*., 2008; Iqbal *et al.*, 2014). Negative regulators of this interaction are the phosphatase and tensin homolog (PTEN) and inositol polyphosphate 4- phosphatase type II (INPP4B) which de-phosphorylate phosphotidylinositol-3,4,5-trisphosphate (PIP_3) to phosphotidylinositol-4,5-bisphosphate (PIP_2) and then to phosphotidylinositol-4-phosphate (PIP). PIP_3 causes translocalisation of AKT to the plasma membrane, hence, its activation (Dittrich *et al.*, 2014) (Fig. 3).

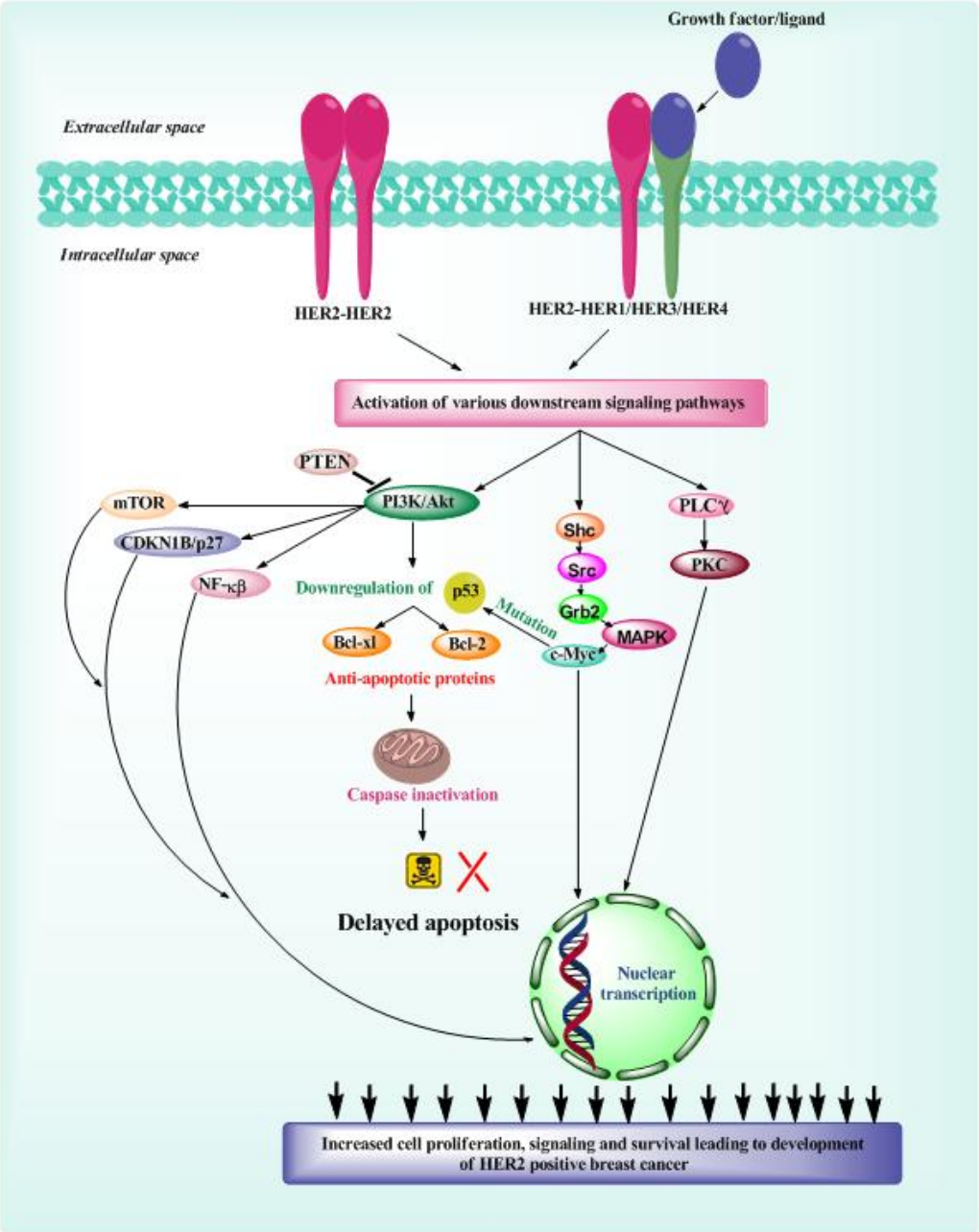


Fig. 3: HER2 homodimerisation or heterodimerisation, activating a number of downstream signaling pathways such as PI3K pathway, MAPK pathway and PLCγ pathway that ultimately leads to enhanced cell proliferation and signaling, delayed apoptosis, hence, contributing towards HER2 positive carcinogenesis

2.8.2. MAPK pathway

One of the most important pathways activated by HER2 signaling is the MAPK pathway. This pathway has been linked to many conditions including breast cancer, as it regulates cell growth, survival, proliferation and migration. The first step in the pathway involves docking proteins such as growth factor receptor-bound protein 2 (GRB2) binding to the newly phosphorylated C-terminus of

HER2. The adaptor protein son of sevenless (SOS) is activated by binding to GRB2. SOS can then cause the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) on RAS. Activated RAS then initiates the activation of a kinase cascade culminating in the phosphorylation and activation of extracellular signal-regulated kinases 1 and 2 (ERK1, ERK2) (Dittrich *et al.*, 2014).

Activated ERK proteins are able to phosphorylate a number of transcription factors those regulate the expression of genes involved in cell growth, differentiation, proliferation, survival, migration as well as transcript turnover and proteolytic activity (Dittrich *et al.*, 2014). MAPK kinase also acts upon c-Myc (gene encoding for transcription factor) producing mutated p53 and hence, increased cell proliferation (Park *et al.*, 2008). Apart from this nuclear transcription factor upregulation is brought about by PLC (Iqbal *et al.*, 2014) and protein kinase C (PKC) activation (Zhou *et al.*, 2013) (Fig. 3).

2.8.3. HER2 cross-talk

Interactions of HER2 with its own family members and with other related family members have been found to be implicated in multiple-pathway activation leading to mammary carcinogenesis and contributing towards drug resistance (Bender *et al.*, 2008). HER2 is the preferred dimerization partner for all other HER-family members, which upon dimerization promotes processes of cellular proliferation, survival, migration, invasion, and differentiation (Graus-Porta *et al.*, 1997). PI3K pathway is the critical signaling pathway for HER2 heterodimerization, and the PIK3CA mutations have been reported to promote breast carcinogenesis (Chakrabarty *et al.*, 2010; Hanker *et al.*, 2013). Resistance towards endocrine agents in ER+/HER2+ breast cancers is seen due to HER2/ER cross talk (Osborne *et al.*, 2003; Shou *et al.*, 2004; Arpino *et al.*, 2008). However, the exact mechanism behind the endocrine resistance is not known. HER2 also shares homology with related family member insulin-like growth factor-I receptor (IGF-1R) and cross talk with IGF-1R confers resistance towards trastuzumab, a monoclonal antibody targeting HER2 (Nahta *et al.*, 2005).

Co-expression of HER2 and adipocyte-secreted factor, leptin has also been reported to contribute to enhanced HER2 signaling and reduced sensitivity to the anti-HER2 treatments (Fiorio *et al.*, 2008). Leptin exposure causes

upregulation of heat-shock protein 90 (Hsp90) promoter activity in the breast cancer cells, leading to enhanced HER2 levels (Giordano *et al.*, 2013). This molecular mechanism provided insights into the associations between obesity and breast cancer progression. Androgen receptor stimulates testosterone secretion. HER2 cross-talk with androgen receptor is responsible for the driving cell proliferation in males (Naderi *et al.*, 2008). Epithelial protein-tyrosine phosphatase 1B (PTP1B) has also been reported to contribute to tumor onset, but it is not required for the tumor maintenance (Balavenkatraman *et al.*, 2011).

Heat-shock protein 72 (Hsp 72) promotes HER2 induced tumorigenesis via upregulation of p21 and downregulation of survivin (inhibitor of apoptosis, IAP) in Hsp 72 knock-out mouse models (Meng *et al.*, 2011). Heat-shock transcription factor 1 (HSF1) is crucial for the maintenance of HSPs and plays a significant role in promoting HER2-induced tumorigenesis (Meng *et al.*, 2010). Mucins, MUC1 and MUC4, activate HER2 by different mechanisms, thus disrupting the cell polarity. MUC1 promotes heregulin-induced HER2 signaling while MUC4 has EGF-like domains on which HER2 can bind and promote tumorigenesis (Kufe, 2009). MUC4, a highly O-glycosylated membrane protein is a putative partner of HER2 and is found to be overexpressed in the trastuzumab-resistant cells (Vu *et al.*, 2012).

2.9. Role of genomic alterations in HER2 positive breast carcinogenesis

2.9.1. Co- amplification of other genes along with HER2

There are many genes in addition to HER2 located on the HER2 amplicon (17q12-21) which may be co-amplified with HER2 as a result of the amplification of the region and in turn, play a crucial role in HER2 positive carcinogenesis. Six genes namely HER2, GRB7, metastatic lymph node 64 (MLN64), phenylethanolamine N-methyltransferase (PNMT), MGC9753 and MGC14832 (Kant *et al.*, 2013; Nguyen *et al.*, 2015) have been reported to be overexpressed in HER2 positive tumors as a result of amplification.

Katz *et al.* indicated that migration and invasion enhancer 1 (MIEN1/C35) is an important oncogene located on HER2 amplicon, whose mRNA expression was found to be associated with the HER2 amplification and it is activated through downstream Syk (Spleen tyrosine kinase) signaling (Katz *et al.*, 2010). Li *et al.* reported that mH2A1.2 isoform of macrohistone 2A1 (H2AFY; mH2A1)

activates HER2 transcription that significantly increases the HER2 expression and cell proliferation (Li *et al.*, 2012). Press *et al.* reported the HER2/ Topoisomerase-2-alpha (TOP2A) co-amplification in a number of breast cancer patients and concluded that it is correlated with the long-term survival in patients, when anthracycline-based chemotherapy was adopted (Press *et al.*, 2011). The co-silencing of MLN64, GRB7, proteasome 26S subunit, non-ATPase 3 (PSMD3) or Post-GPI Attachment To Proteins 3 (PGAP3/ PERLD1) along with HER2 has been reported to result in enhanced inhibition of cell viability, proliferation and induced apoptosis, indicating essential role of these genes in HER2 positive breast carcinoma (Sahlberg *et al.*, 2013).

Apart from this, HER2 amplified breast cancers possess distinct clinical behavior, such as increased sensitivity towards certain chemotherapeutic agents and relative resistance towards hormonal therapy (Gutierrez *et al.,* 2011). This distinct clinical behavior of HER2 amplified breast carcinomas is attributed to co-amplification of the TOP2A gene, located on chromosome 17 which is nearby HER2 gene locus (Gutierrez *et al.,* 2011).

2.9.2. HER2 gene variations

Allelic variations at the proto-oncogene HER2 have been incriminated in the neoplastic process of breast carcinogenesis (Xie *et al.*, 2000). Mutations and single-nucleotide polymorphisms (SNPs) of HER2 contribute towards breast carcinogenesis. The germinal polymorphism at the codon 655 (Val655Ile) (GTC/valine to ATC/isoleucine) (rs1136201), located in the transmembrane domain of the HER2 protein is most commonly explored (Beauclair *et al.*, 2007). This variant has been found to be implicated in the increased autophosphorylation and tyrosine kinase activation (Fleishman *et al.*, 2002). The distribution of this polymorphism was found to vary significantly among different ethnic groups/races (Ameyaw *et al.*, 2000; Ameyaw *et al.*, 2002; Papadopoulou *et al.*, 2006; Kara *et al.*, 2010). Zheng *et al.* conducted a small case-case study and suggested the role of V655I polymorphism in the amplification of HER-2 oncogene (Zheng *et al.*, 2001). However, the specific functional importance of this polymorphism has not been addressed, till date (Montgomery *et al.*, 2003).

Xie *et al.* first identified Val655Ile polymorphism and its association with an increased susceptibility to breast cancer specifically for the early-onset of the

disease, amongst the Han-Chinese women. In this study, It was found that the women possessing Ile/Val or Val/Val genotype displayed more pronounced association as compared with the women possessing Ile/Ile genotype, hence, suggesting the importance of Val allele as an important marker of genetic susceptibility towards breast cancer (Xie *et al.*, 2000). In another study conducted on Shanghai women, Xie *et al.* supported the previously investigated association of HER2 polymorphism with the early-onset of breast cancer (Xie *et al.*, 2001). Wang-Gohrke *et al.* reported HER2 Val allele to be a risk factor for breast cancer among German-Caucasian women, provided the patients had a first-degree family history of breast cancer (Wang-Gohrke *et al.*, 2001). However, in this study, a lack of association was observed between early age and elevated risk of breast cancer contrary to the Han-Chinese women study (Xie *et al.*, 2000).

In another study conducted by Montgomery *et al.* among Australian women, an association was found between V655I polymorphism, particularly, homozygosity for Val allele and increased breast cancer risk in the women below 40 years of age (Montgomery *et al.*, 2003). The more pronounced effect of HER2 polymorphism at a younger age in US women was not confirmed by Hauptmann *et al.* (Hauptmann *et al.*, 2003). Millikan *et al.* conducted their study amongst the African Americans and Whites and found a modest positive association of HER2 Val allele with increased breast cancer risk, particularly in young women and in women with a family history of breast cancer (Millikan *et al.*, 2003). These results were further supported by another study conducted in Ashkenazim (Rutter *et al.*, 2003). Studies carried out among the Portuguese women also confirmed the association of HER2 Val allele and the elevated risk of breast cancer (Pinto *et al.*, 2004). The study conducted among a population of Slovakia, also supported the association of HER2 Val allele with the increased breast cancer risk (ŽÚBOR *et al.*, 2006).

Nelson *et al.* reported an association of HER2 Val allele polymorphism with a reduced risk among older white women from the mid-western United States having no family history of breast cancer (Nelson *et al.*, 2005). Puputti *et al.* reported the over-representation of HER2 Val allele in HER2 positive sporadic breast cancer cases of Finland (Puputti *et al.*, 2006). Papadopoulou *et al.* reported HER2 Val containing genotypes, being more frequent among Greek

Muslim population as compared with the Greek Christian population, hence, presenting Val allele as a modest risk factor for the breast cancer risk (Papadopoulou *et al.*, 2006). Tomassi *et al.* confirmed the positive association of HER2 Val allele polymorphism with both, early onset of breast cancer and family history of breast cancer in a Turkish population (Tommasi *et al.*, 2007). Lee *et al.* also reported the Val carrier association with elevated breast cancer risk in Taiwanese women and the association, being, more apparent among younger women (Lee *et al.*, 2008), also supported by another study conducted among Asian population (Tao *et al.*, 2009). However, in the study carried out amongst a Caucasian population of Italy, HER2 Val allele polymorphism was suggested to be a significant susceptibility biomarker for increased breast cancer risk among older women (Mutluhan *et al.*, 2008).

Some other studies conducted among Arabic, Egyptian, Caucasian, Tunisian, African, Asian and Mexican ethnic groups have also confirmed the association of HER2 Val allele polymorphism with increased breast cancer risk (Kallel *et al.*, 2010; Kara *et al.*, 2010; AbdRaboh *et al.*, 2013; Chen *et al.*, 2013; Pei *et al.*, 2013; Carrillo-Moreno *et al.*, 2016). However, role of V655I genotype in breast cancer susceptibility remains to be controversial, as conflicting results of having no significant association between HER2 Val allele polymorphism and increased breast cancer susceptibility have been reported in several other studies (Baxter *et al.*, 2001; Keshava *et al.*, 2001; Hishida *et al.*, 2002; Akisik *et al.*, 2004; Kamali-Sarvestani *et al.*, 2004; An *et al.*, 2005; Benusiglio *et al.*, 2005; Cox *et al.*, 2005; Frank *et al.*, 2005; Kalemi *et al.*, 2005; Kara *et al.*, 2010; Balavenkatraman *et al.*, 2011; Dahabreh *et al.*, 2011; Sezgin *et al.*, 2011; Nassef *et al.*, 2014; DeAlmeida *et al.*, 2018). In a study conducted among Turkish population, Ile/Ile genotype and Ile allele of HER2 V655I was found to be associated with the increased breast cancer risk (Ozturk *et al.*, 2013). The racial differences contribute to the inconclusive association of HER2 Val allele polymorphism with the breast cancer risk. Although many studies support the role of HER2 Val allele polymorphism with breast cancer susceptibility, there is hardly any data available, regarding its association with the prognosis of the disease. Ozturk *et al.* have reported no significant association of HER2 Val allele polymorphism with the breast cancer prognosis in a study conducted among

Turkish population (Ozturk *et al.*, 2013). Krishna *et al*. performed a meta-analysis of 35 case-control studies and observed significant association of Her2 V655I polymorphism with breast cancer susceptibility in African and Asian populations, but not in other ethnic groups (Krishna *et al*., 2018).

Apart from the dominant HER2 Val655Ile polymorphism, some other HER2 polymorphisms have also been explored. Kallel *et al.* reported the association of a novel dinucleotide repeat H(AC)I4 in intron 4 of HER2 gene with the breast cancer risk, while no association with the breast cancer risk for another single-nucleotide polymorphism (SNP) rs903506 was found in a Tunisian population study (Kallel *et al.*, 2010). Wang *et al.* confirmed no association between HER2 Ala1170Pro polymorphism (rs1058808) and breast cancer risk (Wang *et al.*, 2013), supporting previous studies (Benusiglio *et al.*, 2005; Frank *et al.*, 2005). However, Furrer *et al.* have reported the association of A1170P polymorphism with the breast cancer prognostic factors in non-metastatic HER2-positive breast cancer patients (Furrer *et al.*, 2013), supporting a prior haplotype analysis that revealed a possible candidature of A1170P in inducing functional polymorphism affecting gene expression and/or breast cancer aggression (Cox *et al.*, 2005). Cresti *et al.* have reported the association of A1170P with the enhanced frequency of HER2 overexpression (Cresti *et al.*, 2016). In a haplotype analysis, no associations with breast cancer disposition were found for common HER2 polymorphisms *viz.* rs4252596 (START-657), rs2952155 (intron 1), rs1810132 (intron 4), rs1801200 (exon 17), rs1058808 (exon 27) (Benusiglio *et al.*, 2005). However, Han *et al.* reported a better prognosis and lower HER2 overexpression in subjects lacking the most common haplotype in HER2 gene, i.e., [(C)-(G)-(A)-A-C-(C)].

Besides, this study also suggested the association of two promoter region SNPs in the 5'-untranslated region of HER2 gene with the gene expression and/or breast cancer aggression (Han *et al.*, 2005). Frank *et al.* identified a rare HER2 variant Ile654Val, associated with an elevated risk of familial breast cancer (Frank *et al.*, 2005). However, further studies are required to explore the role of this rare variant as an oncogenic variant, so as to propound its relationship with the prognosis of breast cancer. Apart from the above studies, Su *et al.* have recently reported the association of HER2 rs1058808 and rs2517956

polymorphisms with its increased protein expression in breast cancer, thus, suggesting that HER2 allelic variations play an indispensable role in human breast carcinogenesis (Su *et al.*, 2015).

2.9.3. Other gene variants

Fasching *et al.* have reported the association of SNPs rs700519 and rs4646 of the aromatase gene CYP19A1, with a lower percentage of HER2 positive breast tumors in a German-cohort analysis (Fasching *et al.*, 2008). Kallel *et al.* reported that ESR1 T594T GG genotype is associated with the higher expression of HER2, suggesting an important role of ESR1 gene polymorphisms in the pathogenesis of HER2 positive breast cancers in Tunisian population (Kallel *et al.*, 2009). Surekha *et al.* reported an association of CYP1A1*2 (Ile462Val) polymorphism with breast cancer, in which a higher frequency for heterozygous Val allele was observed for HER2 positive cases in the South Indian population (Surekha *et al.*, 2009). These researchers also reported an increased A2 genotype frequency of CYP17 gene in HER2 positive breast cancer cases (Surekha *et al.*, 2009). Cerne *et al*. found the association of KRAS variant rs61764370 with HER2 overexpression in a Caucasian population (Cerne *et al*., 2012). The KRAS variant has been found to act by disrupting a let-7 mi-RNA binding. The KRAS variant, located in the 3'-untranslated region resulting from a T- to G- base substitution, leads to increased KRAS expression and lowered let-7 levels (Chin *et al*., 2008; Smits *et al*., 2011). In Czech population study, Uvirova *et al*. depicted that G-allele of the KRAS polymorphism (TG/GG genotype) may lead to alteration in HER2 gene expression profile i.e. G-allele may act as a potential genetic marker for the development of HER2 negative breast cancer (Uvirova *et al*., 2015).

Maae *et al*. reported the association of VEGFA -2578CC, -634CC and -7CC genotypes with worse prognosis in the primary HER2 positive breast cancer patients of Denmark (Maae *et al*., 2012), supporting a previous study that depicted a positive association between HER2 and VEGF expression in a population of USA (Konecny *et al*., 2004). However, in multivariate analysis, only VEGFA -634G>C was found to act as an independent prognostic factor (Konecny *et al*., 2004). Pei *et al.* indicated an association of BRCA1, K1183R AA

genotype with favourable survival in the HER2 positive Chinese familial breast cancer patients (Pei *et al.*, 2013).

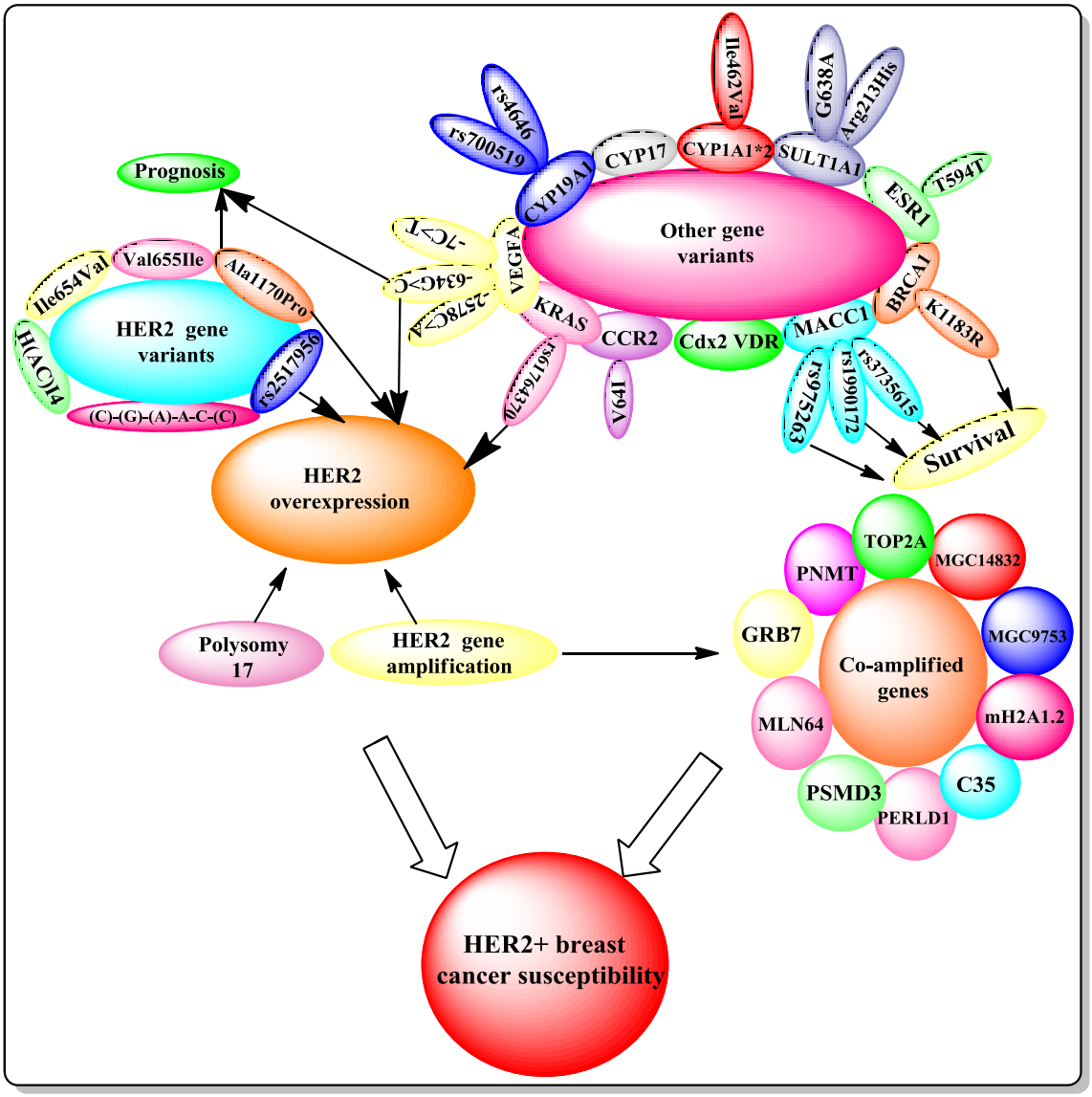


Fig. 4: Genomic alterations implicated in HER2 positive breast carcinogenesis, which include mainly HER2 overexpression during which many genes along with HER2 get amplified, HER2 and other gene variants. All these genetic alterations are either affecting prognosis or overexpression, finally leading to enhanced susceptibility towards HER2 positive breast cancer

Muendlein *et al.* observed a significant positive impact of MACC1 polymorphisms (rs1990172, rs975263, and rs3735615) on survival in HER2-positive breast cancer patients of Austria. This study depicted linkage between rs1990172 and rs975263; and the damaging effect of rs3735615 on MACC1 protein. Damaged MACC1 protein limited hepatocyte growth factor receptor (HGFR/ c-MET) expression and, hence, restricting the metastatic potential of cancer cells (Muendlein *et al.*, 2014). Sulfotransferases activate estrogens and

Ottini *et al.* have reported a significant association of SULT1A1 Arg213His polymorphism with HER2 positive male breast cancer patients in Italy (Ottini *et al.*, 2014). Savukaitytė *et al.* reported another polymorphism of SULT1A1, i.e., G638A AA genotype in positive association with HER2 positive breast cancer risk in Lithuanian population (Savukaitytė *et al.*, 2015). VDR, a nuclear transcription factor, plays a crucial role in cell-cycle regulation, differentiation, apoptosis and, synchronizes the action of hormone responsive genes. Pulito *et al.* found an association of Cdx2 VDR polymorphism, particularly AA genotype in Italian HER2 positive breast cancer patients (Pulito *et al.*, 2015). Chemokine receptors play a crucial role in the tumor progression. Banin-Hirata *et al.* reported a plausible involvement of CCR2, V64I allelic variant in HER2 positive breast cancer (Banin-Hirata *et al.*, 2015). Park *et al.* found a significant association of CASP8 variant rs2293554 with HER2-positive breast cancer (Park *et al.*, 2016). Different genes and their variants that are contributing towards HER2-positive breast cancer susceptibility have been illustrated in Fig. 4.

2.10. HER2-targeting agents for the treatment of HER2-positive breast cancer

A dramatic improvement in the clinical outcome of HER2-positive breast cancer patients has been seen, since the development of HER2-targeting agents. There are two mechanistically distinct approaches for targeting HER2 in breast cancer, i.e. antibody-based therapy and TKIs (Moasser *et al.*, 2015).

2.10.1. Antibody-based therapy

The antibody-based drugs act by selectively target the surface of HER2-overexpressing cancer cells and delivering a variety of cytotoxic or immunologic effectors over there. FDA approved antibody-based treatments for clinical use in HER2-positive breast cancer include monoclonal antibodies (trastuzumab, pertuzumab and trastuzumab-biosimilars (trastuzumab-dkst, trastuzumab-pkrb and trastuzumab/hyaluronidase-oysk); and an antibody-drug conjugate (ado-trastuzumab emtansine) (Moasser *et al.*, 2015).

2.10.2. Tyrosine-kinase Inhibitors (TKIs)

Targeting HER2-positive breast cancer using HER2 TKIs, is apparently a highly rational approach. HER2 TKIs inhibit the catalytic HER2 kinase domain and therefore, HER2-driven signaling. Till date, there two HER2 TKIs i.e., lapatinib

and neratinib those have been approved by FDA for the treatment of HER2-positive breast cancer (Moasser *et al.*, 2015).

2.11. Trastuzumab, the first milestone: Success and limitations

Trastuzumab (Herceptin, Genentech Inc.) is the HER2 targeted monoclonal antibody, approved by Food and Drug Administration (FDA) in 1998 for the first-line treatment of metastatic breast cancer. Trastuzumab is recommended for use as a monotherapeutic agent as well as combined with chemotherapeutic agents for the treatment of HER2 positive breast cancer. It was developed by Genentech Inc (San Francisco, CA, USA). Trastuzumab works by acting against the extracellular domain IV of HER2 (Singla *et al.,* 2017).

Several mechanisms underlying the therapeutic effect of trastuzumab have been elucidated, till date. These mainly include HER2 internalization and degradation; inhibition of proteolytic cleavage of HER2 ECD and thus, preventing p95HER2 formation; antibody-dependent cellular cytotoxicity; inhibition of HER2-HER3 heterodimerization and; the disruption of PI3K/Akt and MAPK pathways (Vu *et al.*, 2012; Maximiano *et al.,* 2016). Of these, ADCC has been regarded as the principle factors contributing towards the trastuzumab-efficacy.

Trastuzumab along with other chemotherapeutic agents has been reported to boost response rate, progression-free survival and overall survival in patients with metastatic HER2 positive breast cancer as compared to chemotherapy alone. It reduced the risk of disease relapse and prolonged survival in patients with operable, HER2-positive breast cancer. Despite these achievements, the majority of the patients develop resistance to this monoclonal antibody (Singla *et al.*, 2018).

2.11.1. Mechanisms of resistance to trastuzumab

There are two kinds of resistance treatment resistance; one is primary or inherent resistance and the other is secondary or acquired resistance (Maximiano *et al.,* 2016). About 65% of the HER2 positive breast cancer patients develop primary resistance i.e., they don’t respond to the initial trastuzumab therapy. Whereas, approximately 70% of them develop secondary resistance to trastuzumab i.e., after an year of initial treatment those show disease-progression (Maximiano *et al.,* 2016). HER2 mutations and other intracellular

alterations related to signaling pathway are critically involved in conferring resistance to trastuzumab (Singla *et al.,* 2017).

2.11.2. Role of genomic alteration in determining response towards trastuzumab-therapy

Genetic variants play a critical role in predicting response to HER2 targeted trastuzumab therapy.

2.11.2.1. HER2 gene variants

HER2 gene variants have been studied for their association with response to anti-HER2 therapeutics. Specially, HER2–V655I genotype is the most explored in association with breast cancer risk as well as the response to trastuzumab treatment. It constitutes a risk factor for trastuzumab-induced cardiotoxicity, besides contributing towards sensitivity towards trastuzumab treatment.

Beauclair *et al.* found that the cells expressing Val allele show sensitivity towards trastuzumab treatment. However, as far as cardiac dysfunction is concerned, it has been observed only in heterozygous Ile/Val subjects and not in Val/Val homozygous subjects. But, no interpretation regarding a possible role for the Val allele as a predisposing factor for cardiotoxicity can be made, as there were only 4 Val/Val homozygous subjects (Beauclair *et al.*, 2007). Roca *et al.* found an association between the HER2–V655I and cardiac toxicity (Roca *et al.*, 2013), the data were also confirmed by Lemieux *et al.* (Lemieux *et al.*, 2013). Han *et al.* found that the alteration of the HER2 gene function by HER2 V655I polymorphism remains confined only to the HER2 positive subjects. The Val variant in HER2 positive patients contributes to the aggressiveness and sensitivity towards trastuzumab treatment. A poor clinical outcome was observed in HER2-positive patients with a Val variant when treated with adjuvant therapy without trastuzumab. However, treatment with adjuvant therapy in combination with trastuzumab given to these patients resulted in favourable survival (Han *et al.*, 2014).

Pena *et al.* confirmed the association of HER2 655 A>G (rs1136201) polymorphism with trastuzumab-induced cardiotoxicity in HER2-positive breast cancer patients (Peña *et al.*, 2015). A study showed conflicting results of the association between codon 665 and cardiotoxicity. However, in the same study, P1170A polymorphism was found to be associated with an elevated risk of

cardiotoxicity from trastuzumab therapy and the proline variant was found to be more prevalent than the alanine among the subjects showing trastuzumab-induced cardiotoxicity (Stanton *et al.*, 2015).

Apart from the mutations as mentioned earlier, the truncation of the HER2 molecule itself to produce p95HER2 or carboxy-terminal fragments (CTFs) of HER2 is one of the most potential mechanisms contributing to trastuzumab resistance. The p95HER2 fragments confer resistance to trastuzumab, as these are lacking the trastuzumab-binding portion of the extracellular domain of HER2 (Zagozdzon *et al.*, 2011).

2.11.2.2. Alterations in the PI3K pathway

PI3K plays an essential role in diverse cellular signaling pathways such as proliferation, metabolism, migration, translation, apoptosis avoidance, and angiogenesis (Kalinsky *et al.*, 2009). HER2-PI3K-AKT pathway alterations such as mutation and amplification of PIK3CA, tumor suppressor gene, PTEN loss, AKT1 mutations, extracellular domain-truncatedHER2 (p95HER2) expression, are associated with the poor response towards the trastuzumab treatment in breast cancer patients (Berns *et al.*, 2007). PIK3CA encodes the catalytic subunit of class 1A, a lipid phosphokinase, *i.e.,* p110α. PIK3CA mutations result in increased lipid kinase activity and Akt phosphorylation. 8% to 40% PIK3CA mutation frequency was reported in human breast cancers (Kalinsky *et al.*, 2009). High frequency of PIK3CA mutations have been found in HER2+ breast tumors (Santarpia *et al.*, 2012). Most of the PIK3CA mutations occur at three hotspots: two in exon 9 and one in exon 20 and His1047Arg is the most common mutation, which is located in exon 20 (Castaneda *et al.*, 2014). PTEN negatively regulates the PI3K pathway resulting in decreased Akt activity. Thus, PIK3CA mutations or PTEN loss lead to increased downstream signaling, conferring resistance to trastuzumab (Jensen *et al.*, 2012).

Esteva *et al.* analyzed HER2-overexpressing metastatic breast cancer patients undergoing trastuzumab-based therapy, for four biomarkers *viz.,* PTEN, p-AKTSer473, p-p70S6K-Thr389, and PIK3CA. Out of these, only PTEN alone significantly correlated with the shorter survival times and PTEN loss combined with each of other three biomarkers, *i.e.,* p-AKTSer473, p-p70S6K-Thr389, and PIK3CA, accounted for an increased correlation. PTEN loss and/or PIK3CA

mutations accounted for shorter survival time among the subjects under investigation, hence, depicting a pivotal role of PI3K pathway activation in trastuzumab resistance (Esteva *et al.*, 2010). Jensen *et al.* supported the association of PIK3CA mutations or increased PI3K pathway activity with significantly poorer survival among patients receiving trastuzumab-based therapy (Jensen *et al.*, 2012). Takada *et al.* concluded that the gene alterations in the PI3K and ER (estrogen receptor) pathway were associated with the poor survival among HER2 positive patients along with a positive status for hormone receptor; receiving neoadjuvant chemotherapy with trastuzumab. Pathological complete response is less likely to be achieved among HER2-positive breast carcinoma patients with a PIK3CA mutation to whom neoadjuvant anthracycline-taxane–based chemotherapy plus anti-HER2 treatment was given (Takada *et al.*, 2013). Adamczyk *et al.* suggested the involvement of androgen receptor along with PTEN in conferring sensitivity towards trastuzumab-based treatment (Adamczyk *et al.*, 2015).

2.11.2.3. Fc-gamma receptor polymorphisms

Trastuzumab exerts its action through antibody-dependent cell-mediated cytotoxicity (ADCC), and the notion is supported by several studies (Clynes *et al.*, 2000; Carson *et al.*, 2001; Gennari *et al.*, 2004; Arnould *et al.*, 2006). Fc-gamma receptor (FcgR) is involved in the ADCC effect of trastuzumab. Thus, the FcgR polymorphisms affect the response of the patients towards trastuzumab.

Musolino *et al.* evaluated HER2 positive breast cancer patients for the FcgR IIIa-158 valine(V)/phenylalanine(F), FcgR IIa-131 histidine(H)/arginine(R), and FcgRIIb-232 isoleucine(I)/threonine(T)polymorphisms and found an association of FcgR IIIa-158 V/V and FcgR IIa-131 H/H genotype with objective response rate (ORR) and progression-free survival (PFS). Peripheral blood mononuclear cells (PBMCs) harboring V/V and/or H/H genotype had a significantly higher trastuzumab-mediated cytotoxicity than the PBMCs harboring different genotypes (Musolino *et al.*, 2008).

Tamura *et al.* found an association between FcgRIIA-131 H/H polymorphism with the pathological response to trastuzumab-based neoadjuvant chemotherapy in the early-stage breast cancer and the objective response to trastuzumab in metastatic breast cancer. However, the FcgRIIIA-158 V/V

genotype did not correlate with the pathological response, but exhibited a tendency to be associated with the objective response (Tamura *et al.*, 2011). Hurvitz *et al.* reported no correlation between FcgRIIIA-V/F and FcgRIIA-H/R SNPs and DFS in HER2 positive patients treated with trastuzumab. The discrepancies from previous studies may be due to differences in the intrinsic population factors or chemotherapy regimens (Hurvitz *et al.*, 2012). Roca *et al.* reported a significant correlation of FcgRIIA-131 H/H genotype with a shorter survival rate and no correlation of FcgRIIIA-158 V/V genotype with survival in trastuzumab-treated patients (Roca *et al.*, 2013). Norton *et al.* identified no genetic association of FcgRIIA and FcgRIIIA polymorphisms with superior DFS in patients treated with trastuzumab (Norton *et al.*, 2014).

2.11.2.4. Other genes involved in prediction of response towards trastuzumab-therapy

MET oncogene, localised on chromosome 7 encodes for the dimeric tyrosine kinase receptor for hepatocyte growth factor (HGF) and is involved in the cell proliferation, survival, and angiogenesis. Minuti *et al.* found a correlation between the MET and HGF overexpression with short relapse-free and overall survival (OS) in HER2 positive metastatic breast cancer patients undergoing trastuzumab-based treatment (Minuti *et al.*, 2012). MYC is a commonly amplified oncogene with diverse functions in the cell signaling and is associated with both poor and good prognoses (Liao *et al.*, 2000; Lutz *et al.*, 2002). National Surgical Adjuvant Breast and Bowel Project (NSABP) B31 trial suggested the association of MYC/HER2co-amplification with additional benefit from adjuvant trastuzumab in patients with early-stage breast cancer. Perez *et al.* investigated an association between the MYC amplification and DFS in a similar adjuvant trastuzumab HER2-positive breast cancer trial—North Central Cancer Treatment Group (NCCTG) N9831, finding no association between the MYC amplification and additional trastuzumab benefit (Perez *et al.*, 2011).

Tristetraprolin (TTP), also known as zinc finger protein 36(ZFP36), is the memberof a family of three human genes (ZFP36, ZFP36L1, andZFP36L2), characterized by two tandemly repeated zinc finger motifs. TTP plays a key role in the inflammation and tumor development. Griseri *et al.* identified a synonymous polymorphism (rs3746083) in TTP showing a statistically significant

association with decreased response towards trastuzumab in HER2-positive-breast cancer patients (Griseri *et al.*, 2011). Various genomic alterations predicting response towards trastuzumab treatment have been summarized in Fig. 5. Besides, there are several other biomarkers that are implicated in development of resistance against trastuzumab, which have been reviewed (Menyhárt *et al.*, 2015, Maximiano *et al.,* 2016).

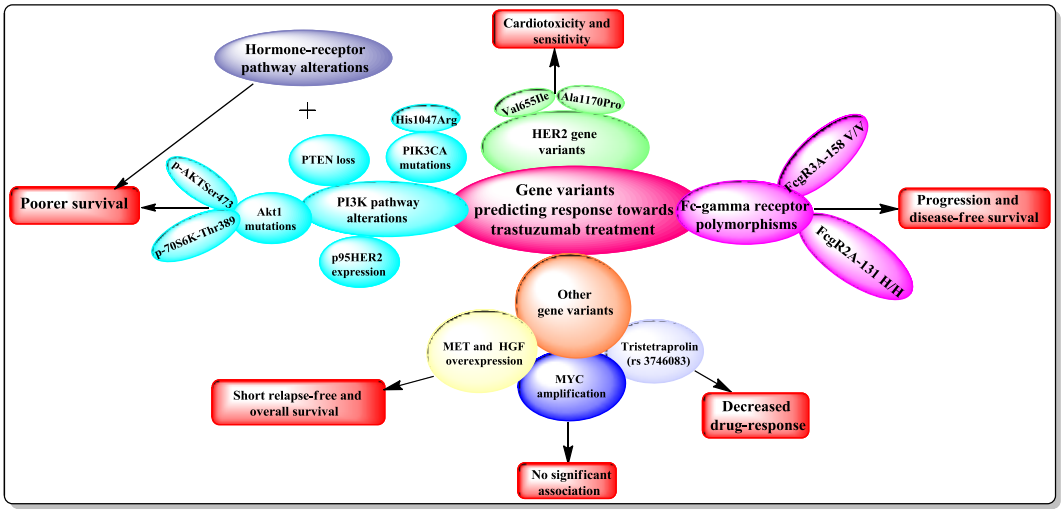


Fig. 5: Gene variants in association with response elucidation towards trastuzumab-treatment. The major gene alterations among them include PI3K pathway alterations, Fc-gamma receptor polymorphisms and *HER2* gene variants. Apart from them, other gene variants that have been shown in the figure may also make a contribution towards the process

2.12. Other antibody-based therapies and the associated concerns

Trastuzumab binds to the extracellular domain IV of HER2 and prevent HER2 homodimerization and activate cell-signaling pathway (Vu *et al.*, 2012). On the other hand, pertuzumab binds to the extracellular domain II of HER2, and inhibit HER2/HER3 heterodimerization and prevent anti-apoptotic PI3K/Akt signaling (Capelan *et al.*, 2013). Both of these drugs bind to the extracellular domain and are associated with the adverse drug reactions including cardiotoxicity. It has been observed that trastuzumab exhibited more cardiotoxicity as compared to pertuzumab (Capelan *et al.*, 2013). The development of *de novo* resistance against antibody therapy is the major problem encountered in most of the cases (Hurvitz *et al.*, 2013). Thus new class of drugs derived from antibodies i.e. antibody-drug conjugate have been developed in order to potentiate effective drug targeting. Trastuzumab-emtansine (T-DM1) is an approved antibody-drug conjugate for treating breast carcinoma patients with HER2 overexpression

(www.fda.gov) (Amiri-Kordestani *et al.*, 2014). Trastuzumab linked to the microtubule-depolymerizing agent and it facilitates the delivery of cytotoxic agent DM1 to HER2 overexpressing cancer cells (Verma *et al.*, 2012). However, the adverse drug reactions remained major issue with this class of drugs too (Amiri-Kordestani *et al.*, 2014).

2.13. HER2 TKIs: A new hope

TKIs are the small-molecules competing for the ATP binding site of the intracellular catalytic domain of kinase. TKI binding inhibit protein phosphorylation and disturb the signal transduction (Moasser *et al.*, 2015). Lapatinib (GW572016/Tykerb) is the only small-molecule TKI approved by FDA for the treatment of metastatic breast cancer overexpressing HER2 (www.fda.gov). Lapatinib is orally bioavailable and possesses reduced risk of adverse cardiac events as compared to the intravenous administration of antibodies. It effectively inhibits trastuzumab-resistant truncated HER2 protein, p95HER2 (Xia *et al.*, 2004). Lapatinib can also cross blood-brain-barrier (BBB) while monoclonal antibodies cannot cross it. Hence, small-molecules may prove effective treatment strategy for curing central nervous system metastases in HER2 overexpressing breast cancer patients (Lin *et al.*, 2008). Despite the advantages of lapatinib, the problem of resistance-development towards it has also been observed (Hurvitz *et al.,* 2013). Moreover, significantly improved survival with T-DM1 as compared to lapatinib has been observed in HER2 positive breast cancer. This indicates the superiority of antibodies over TKIs (Verma *et al.*, 2012). The compensatory upregulation of HER3 in response to HER2 inhibition by TKIs mainly accounts for the resistance-acquisition and lower clinical efficacy. Hence, the status of HER2 TKIs as monotherapeutic agents remains controversial. In that case, combination-therapy may help (Moasser *et al.*, 2015). Neratinib has been recently approved by FDA, but its clinical scope is limited to early-stage breast cancer (www.fda.gov). Although the TKIs were developed with much expectations, but antibody-based therapy remains the gold standard for the treatment HER2 overexpressing breast cancer. Thus, still there is need of development of TKIs with high therapeutic potential for effective HER2 inhibition in breast cancer.

2.14. HER2: Crystal structure of the intracellular kinase domain

Aertgeerts *et al.* (Aertgeerts *et al.*, 2011) reported the first high-resolution crystal structure of the kinase domain of HER2 in complex with SYR127063, a pyrrolo[3,2-d]pyrimidine-based potent and selective HER2 inhibitor. A Gly-rich region (Gly776-Ser779) of α-helix C-β4 loop following α-helix C is unique to HER2, providing conformational flexibility within the HER2 active site. It can be accounted for the previously reported low intrinsic activity of HER2 (Cohen *et al.*, 1996; Brignola *et al.*, 2002; Fan *et al.*, 2008). Genetic analyses on cancer patients have showed that the mutations or insertions of hydrophobic residues before Gly776, enhance the catalytic activity of HER2 (Stephens *et al.*, 2004; Shigematsu *et al.*, 2005; Lee *et al.*, 2006; Wang *et al.*, 2006). A comparative study was also conducted involving the crystal structure of HER1 with TAK-285, a dual HER1/HER2 inhibitor containing pyrrolo[3,2-d]pyrimidine-based backbone so as to develop understanding about the key binding interactions that influences the potency and selectivity of HER inhibitors. The two complexes and their crystal structures were found very similar.

The kinase domain crystal structure studies revealed that the dimeric protein is asymmetric and each monomer has been occupied by the inhibitor (Emsley *et al.*, 2010). The HER2 construct taken for structural analysis was a shortened form of human cytoplasmic tyrosine kinase domain, having a part of C-terminal segment. This construct comprises 703–1029 residues and three N-terminal mutations, M706A, Q711L, and M712L were introduced to increase expression as well as to facilitate the crystal formation of the protein-inhibitor complex. A flexible hinge region connects the N-terminal lobe (N-lobe) that contains mostly β-strands and one α-helix; and the C-terminal lobe (C-lobe) that is predominantly α-helical. A deep cleft comprising the ATP binding site separates these two lobes. Most of the residues that are responsible for the catalytic activity were located in proximity to the cleft which include the glycine-rich nucleotide phosphate-binding loop (Leu726–Val734), the α-helix C (αC; Pro761–Ala775) of the N-lobe of the kinase and DFG motif (Asp863–Gly865), the catalytic loop (Arg844–Asn850), and the activation loop (A-loop; Asp863–Val884) of the C-lobe of the kinase. The established feature of the active conformation, i.e. the salt bridge interaction between two highly conserved

residues (Lys753 and Glu770) has not been observed in the crystal structure. The key binding interactions in the ATP binding site and other features like disordered A-loop were highly similar to that observed for HER1-TAK285 complex. Moreover, HER2-SYR127063 salt bridge formation was also not observed for HER1-TAK-285. The central pyrrolo[3,2-d]pyrimidine ring of SYR127063 exhibits H-bonding interactions with amino-acid residues Met801 and Thr862 and hydrophobic interactions in the adenine site. Hydrophobic interactions with amino-acid residues Thr862, Glu770, Met774, Ser783, Leu785, Leu790, Leu796 and Phe864 were displayed by the trifluoromethyl group of SYR127063 (Aertgeerts *et al.*, 2011).

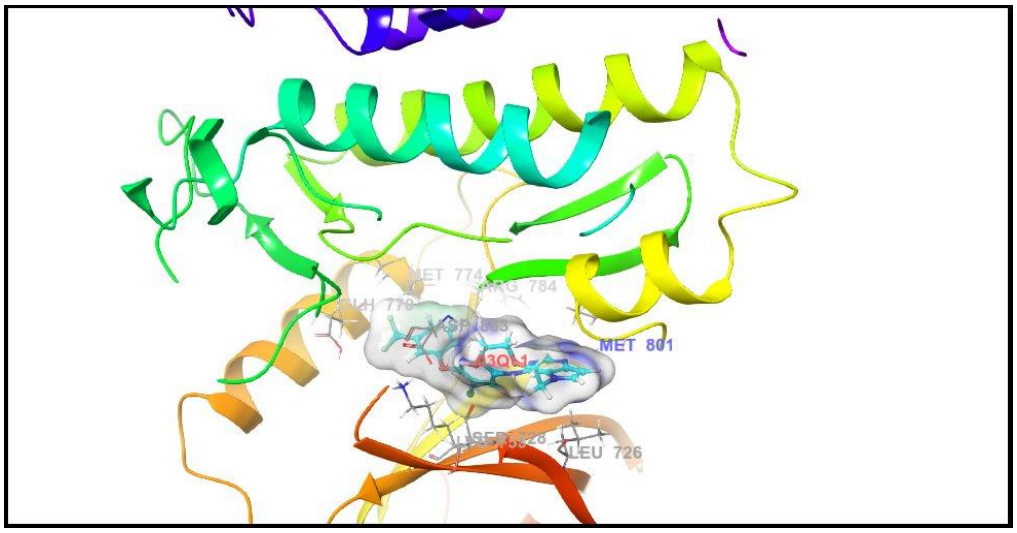


Fig. 6: Active site of HER2 kinase (PDB ID: 3PP0) with co-crystallized ligand SYR127063 (Schrodinger, Maestro 9.0)

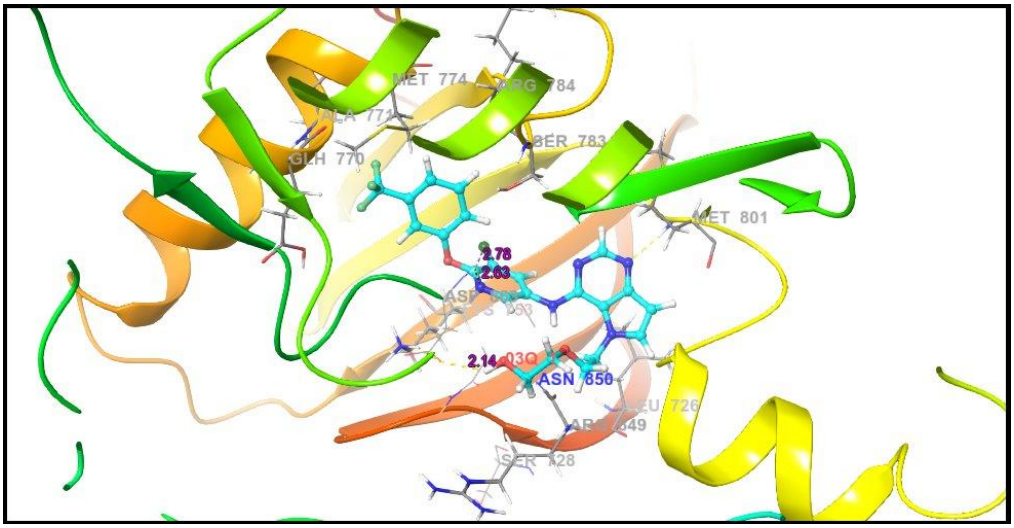


Fig. 7: Expanded view of active site of HER2 kinase (PDB ID: 3PP0) with co-crystallized ligand SYR127063 (Schrodinger, Maestro 9.0)

Aertgeerts *et al.* (Aertgeerts *et al.*, 2011) revealed an allosteric mechanism of action for HER2 which was similar to the activation mechanisms for HER1 and HER2, as reported previously (Zhang *et al.*, 2006; Zhang *et al.*, 2007; Qiu *et al.*, 2008). For HER2, a rotation and translation function similar to asymmetric dimers HER1 and HER4 (Zhang *et al.*, 2006; Zhang *et al.*, 2007; Qiu *et al.*, 2008) was noticed suggesting an allosteric mechanism of activation. Ala705-Thr718 residues in N-terminus, Pro761-Ala775 residues in α-helix C and Leu790-Ser792 residues in loop between β-strand-4 and -5 of one HER2 monomer are involved in interactions with Pro926-Lys937 residues in α-helix G, Gly938-Pro945 residues in loop between α-helix H and α-helix of other HER2 monomer. Mainly closed hydrophobic intermolecular interactions were formed between Lys765, Leu768, Asp769 and Tyr772 residues in α-helix C of one HER2 monomer and C-lobe of kinase domain of other HER2 monomer (Aertgeerts *et al.*, 2011).

We re-docked SYR127063 with the crystal structure of HER2 kinase domain using docking software Schrodinger, Maestro (Version 9.0). The three-dimensional docking structures (Fig. 6 and Fig. 7) showed active site of HER2 kinase domain and ligand-interaction pattern, respectively. From these re-docked structures, we found that the ligand interaction pattern for binding of SYR127063 with HER2 kinase domain was similar as demonstrated previously by Aertgeerts *et al.* (Aertgeerts *et al.*, 2011).

2.15. Developments in the screening of small molecules acting as HER2 TK inhibitors

HER2-targeted antibodies (trastuzumab, pertuzumab) and small-molecule TKIs (lapatinib) are the approved therapies for HER2 positive breast carcinoma (www.accessdata.fda.gov). Trastuzumab exhibits a response rate of only 15-26% among HER2 positive breast cancer patients (Nielsen *et al.*, 2013) and in most of the cases it is ineffective as single agent (Dokmanovic *et al.*, 2013). The development of resistance against antibody-therapy as a result of production of oncogenic truncated form of receptor (p95HER2/HER2Δ16) is the major limitation. Small molecule inhibitors such as lapatinib can inhibit truncated HER2 protein (Xia *et al.*, 2004). As TKIs can cross BBB, hence these can be developed as effective agents for treating brain metastases in HER2 expressing breast

cancer (Lin *et al.*, 2008). However, there are some limitations associated with the currently available TKIs such as toxicity, resistance and poor pharmacokinetic profile. Hence, there is need of developing novel HER2 inhibitors with improved pharmacological profile and devoid of above mentioned limitations.

The role of HER2 TKIs for the treatment of breast cancer has been reviewed by a number of research groups (Spector *et al.*, 2007; Saini *et al.*, 2011; Tsang *et al.*, 2012; Cameron *et al.*, 2013; Gradishar, 2013; Nielsen *et al.*, 2013; Vrbic *et al.*, 2013; Schroeder *et al.*, 2014). Nevertheless, the optimization and SAR studies have not been discussed in detail. Different HER2 TKIs have been classified on the basis of chemical scaffolds such as 4-anilinoquinazolines, pyrrolo-triazenes, pyrrolo and pyrazolo-pyrimidines, thieno-pyrimidines, and N-aryl pyrimidines. 4-anililinoquinazoline is the scaffold possessed by majority of HER2 inhibitors those have been discussed in detail.

2.16. 4-anilinoquinazolines acting as HER2 inhibitors in breast cancer

Lippa *et al.* (Lippa *et al.*, 2007) synthesized a series of selective HER2 kinase inhibitors containing quinazoline and pyrido[4,3-d]pyrimidine scaffolds. The SAR studies for the designed series have been shown in Fig. 8. The compounds **1** and **2** (Fig. 9) were the most potent analogues both inhibiting HER2 kinase and HER2 cells with IC_{50} values below 25 nM. The HCl salt of compound **1** and compound **2** showed low clearance rate of 2.5 ml/min/kg. Compound **1** as HCl salt showed 54% bioavailability while compound **2** exhibited poor bioavailability (Lippa *et al.*, 2007). Rachid *et al.* (Rachid *et al.*, 2009) designed and synthesized combi-molecules by grafting carbamate-substituted monoalkyltriazene moiety to anilinoquinazolines. These molecules were meant for the inhibition of multiple targets in cancer cells. The synthesized combi-molecules were evaluated as HER1 TK and HER2 transfectant against NIH3T3-neu cell line. Compound **3** (Fig. 9) was found to be the most potent with an IC_{50} value of 1.50 µM. The combi-molecules possessed DNA damaging properties too. SAR studies indicated that the replacement of methyl ethanaoate group with chloromethyl and β-nitrorophenyl groups lead to decrease in activity (Rachid *et al.*, 2009).

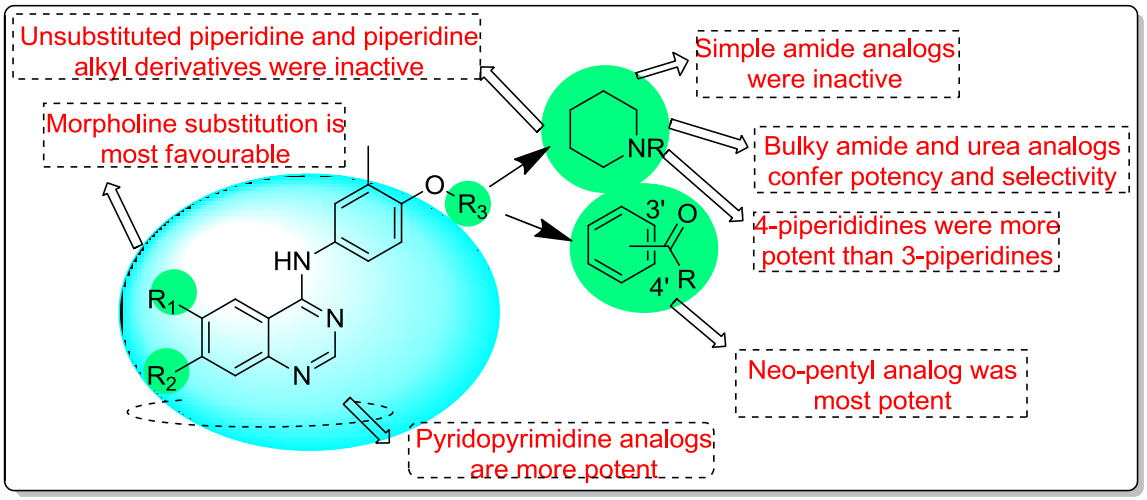


Fig. 8 SAR studies of anilinoquinazolines **(compounds 1-2)**

Mahboobi *et al.* (Mahboobi *et al.*, 2010) combined the structural features of lapatinib with an (E)-3-(aryl)-N-hydroxyacrylamide motif present in histone-deacetylase (HDAC) inhibitors with an aim to produce multi targeting chimeric compounds inhibiting HER1/HER2 TK and HDAC with less likelihood of developing drug-resistance. Among the synthesized chimeric analogues, compound **4** (Fig. 9) was found to be the most potent, exhibiting IC_{50} value below 1 μM against HER1/HER2 kinase, HDAC enzyme and HER2 overexpressing SKBR3 cancer cell line. When the furan ring of **4** was replaced with thiophene or benzene ring, the HDAC inhibition activity reduced while HER1 and HER2 inhibition remain unaffected. The benzamide and hydroxamate analogues obtained *via* replacement of hydroxyacrylamide moiety of **4,** retained the kinase inhibition activity. However, HDAC inhibitory activity diminished in most of the synthesized hybrid analogues (Mahboobi *et al.*, 2010).

Wu *et al.* (Wu *et al.*, 2010) synthesized a series of 4-benzothienyl amino quinazolines as hybrids of HER1 inhibitor, gefitinib. The benzothiophene derivatives attribute to increased cytotoxicity but decreased HER1 inhibition than gefitinib. The most potent compound **5** (Fig. 9) exhibited antiproliferative activity against HER2 transfected MCF-HER2 breast cancer cells with IC_{50} value of 2.7 μM. The secondary amino-substituted propoxy side chain at position 7 in place of position 6 in basic scaffold, led to increased HER2 and MET inhibitory ability with reduced HER1 inhibitory potency (Wu *et al.*, 2010). Beckers *et al.* (Beckers *et al.*, 2012) designed and synthesized chimeric broad-spectrum inhibitors of HDAC

and HER1/HER2. The linkage of N-(3-ethynylphenyl)quinazoline-4-amine moiety of HER1 inhibitor with an N-(2-aminophenyl)-3-(1-sulfonyl-1H-pyrrol-3-yl)acrylamide motif in erlotinib retained the selective HER1/HER2 kinase inhibitory properties, along with HDAC inhibition. Whereas, the attachment of N-(3-ethynylphenyl)quinazoline-4-amine moiety with N-(2-aminophenyl)-4-(oxymethyl)benzamidyl group diminished the HDAC inhibitory activity, while significantly enhanced HER1/HER2 inhibitory activity. In this series, compound **6** (Fig. 9) was the most potent HER2 inhibitor with an IC_{50} value of 39 nM (Beckers *et al.*, 2012).

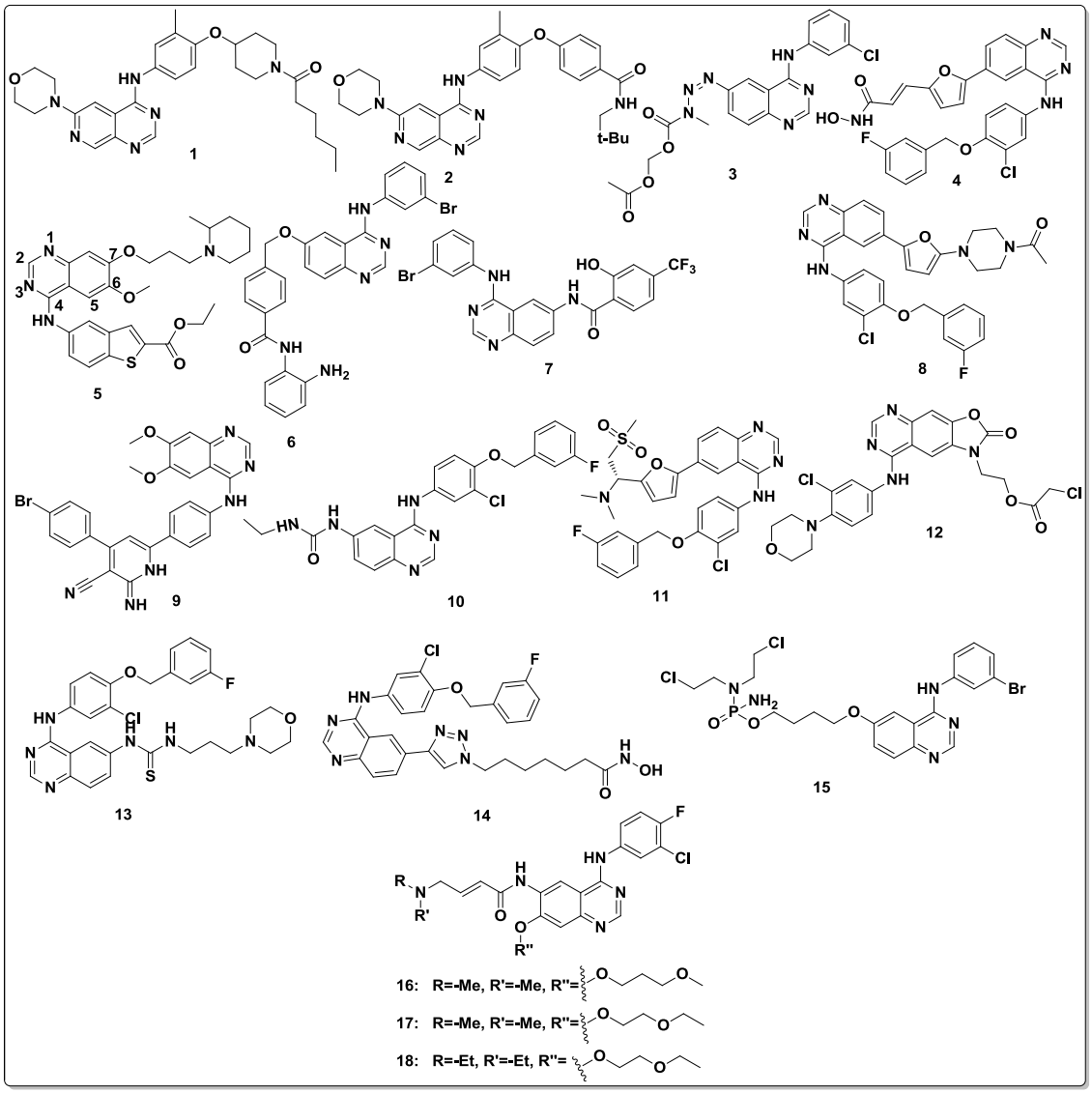


Fig. 9 Structures of anilinoquinazolines acting as HER2 inhibitors

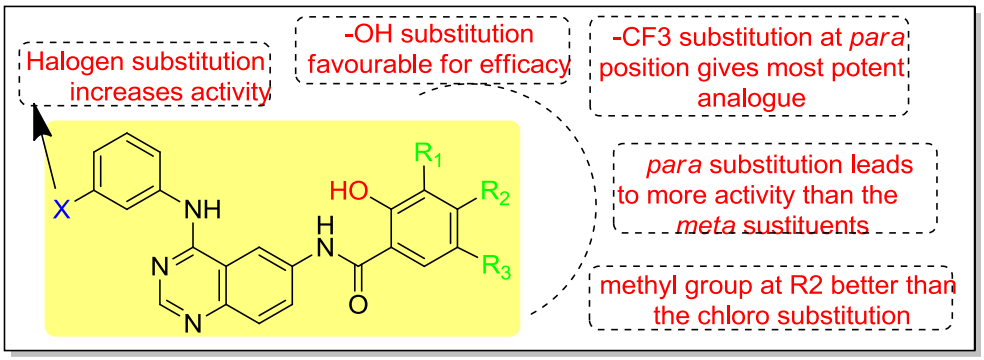


Fig. 10 SAR studies of anilinoquinazolines (compound **7**)

Li *et al.* (Li *et al.*, 2013) designed and synthesized 6-salicyl derivatives of anilinoquinazolines as HER1/HER2 TK inhibitors. All compounds showed moderate to good *in vitro* inhibitory activities in the enzymatic and cellular assays. Compound **7** (Fig. 9) showed most potent and selective dual HER1/HER2 inhibitory activity comparable to that of lapatinib, with IC_{50} values of 120/96 nM. From the molecular docking studies, it was established that the two amino acid residues; SER783 and MET801 located in the hinge-binding pocket were crucial for the activity. *Para* substituted trifluoromethyl group, hydroxy substituent, and ketonic oxygen were crucial for the stabilization of the binding complex. Trifluoromethyl group was involved in the H-bonding interactions with PHE731 and ALA730. SAR studies for compound **7** are summarized in Fig. 10 (Li *et al.*, 2013). Zhang *et al.* (Zhang *et al.*, 2013) designed and synthesized lapatinib hybrids as potent dual HER1/HER2 inhibitors with improved drug like characteristics. Compounds **8** (selatinib, Fig. 9) was found to be the most potent in the series displaying selective inhibitory activity with IC_{50} values of 19.2 nM against BT474 cells and 11.4/6.8 nM against HER1/HER2. Compound **8** showed substantial suppression of tumor growth in NCI-N87 (94.8% inhibition) or SK-OV-3 xenograft (85.7% inhibition) models. Currently, selatinib is under phase I clinical trials (NCT01931943, clinicaltrials.gov) for its pharmacokinetic evaluation in advanced breast cancer subjects (Zhang *et al.*, 2013).

Ahmed *et al.* (Ahmed *et al.*, 2013) carried out combined docking and molecular dynamic simulation studies on anilinoquinazoline-based anti-HER2 ligands. Molecular dynamic simulation studies showed that the compounds form stable complex with the binding site of the HER2 receptor. These compounds displayed various interactions in the hinge region and salt-bridge interactions

with Asp863 and Lys753. Simulation studies revealed the importance of Vander-Waal's interactions for the ligand binding (Ahmed *et al.*, 2013). Ahmed *et al.* (Ahmed *et al.*, 2013) carried out molecular dynamic simulations and binding energy calculations to find out the binding modes and the mechanisms of inhibition of *in silico* designed 4-anilinoquinazoline derivatives against HER1 and HER2. The most active compound showed H-bonding interactions with two amino acid residues, i.e., Asp855 and Lys745 for HER1 and Asp863 and Lys753 for HER2. In addition, ligand showed H-bonding interaction with a conserved Met residue at the hinge region through the N1 atom of the quinazoline ring (Ahmed *et al.*, 2013). Sadek *et al.* (Sadek *et al.*, 2014) designed and synthesized anilinoquinazoline derivatives substituted at 4' position of aniline by bulky arylpyridinyl, arylpropenoyl and arylpyrazolyl moieties. Compound **9** (Fig. 9), was found to be the most potent derivative with IC_{50} value of 1.94 µM, against HER1 and 1.04 µM against HER2. Besides, the compound **9** also exhibited anti-proliferative activity against HER1 overexpressing MDA-MB-231 breast cancer cell lines (Sadek *et al.*, 2014).

Elkamhawy *et al.* (Elkamhawy *et al.*, 2015) designed and synthesized 6-substituted 4-anilinoquinazolines as selective HER1/HER2 TKIs. Most of the compounds exhibited IC_{50} values in nanomolar range against HER1 and/or HER2 kinases. Computational studies indicated that four compounds showed favourable binding interactions at the ATP binding sites of both the kinases. In the series, compound **10** (Fig. 9) displayed promising cytotoxicity with IC_{50} value of 1.82 µM, against BT474 cell line. SAR analysis revealed that the replacement of fluorobenzyloxy moiety with phenoxy group reduced the antiproliferative activity (Elkamhawy *et al.*, 2015). Lyu *et al.* (Lyu *et al.*, 2014) have designed and synthesized a series of lapatinib derivatives by carrying out modifications of straight alkyl side chain of lapatinib into branched ones. These derivatives exhibited significant inhibition of HER1/HER2 as indicated by ELISA assay and western blot analysis. *In vitro* assay showed that these compounds possessed potent cytotoxicity against the HER1/HER2 overexpressing cancer cells. Amongst the synthesized lapatinib derivatives, compound, **11** (Fig. 9) showed most potent antitumor activity, *in vivo,* with an IC_{50} value of 28.8 nM. It has been observed that the IC_{50} value of **11** was two folds higher than that of lapatinib

(IC_{50}= 63.9 nM). Moreover, **11** was found to block the cell cycle progression of BT474 cells in the G_1 phase, causing tumor cell apoptosis. SAR studies indicated that alkylamine substitution such as dimethylamine group on the side-chain was beneficial for HER1/HER2 inhibitory and cytotoxic activity while bulkier substitution led to decreased activity. Further, stereochemical investigations of the compounds showed higher potency for (S)-isomers than for (R)-isomers (Lyu *et al.*, 2014). Yin *et al.* (Yin *et al.*, 2016) developed a series of oxazolo[4,5-g]quinazolin-2(1*H*)-one derivatives as HER1/HER2 TKs inhibitors displaying high activity and low toxicity. Compound **12** (Fig. 9) showed most potent HER1/HER2 kinase inhibition with IC_{50} value of 10/20 nM and displayed IC_{50} value of 0.47µM against SKBR3 cancer cell line. In addition, compound **12** also exhibited anti-proliferative activity against human lung adenocarcinoma cell line (A549). In tumor xenograft models of lung cancer, compound **12** showed higher inhibition efficacy towards tumor growth as compared to lapatinib. N1, N3 atoms in pyrimidine ring and a proton at the anilinic-N position were found to be crucial in this series of compounds. Introduction of electrophilic groups like 2-(2-chloroacetoxy)ethyl and 2-(2-bromoacetyl)ethyl at N-position of oxazolo ring was favourable for HER1/HER2 kinase inhibitory activity. The detailed SAR studies have been illustrated in Fig. 11 (Yin *et al.*, 2016).

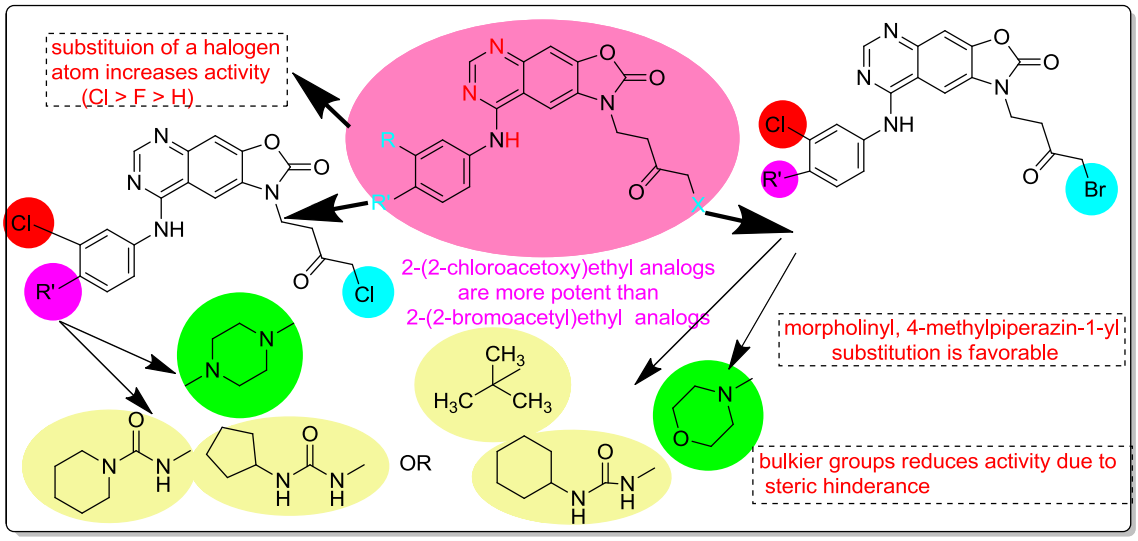


Fig. 11 SAR studies of anilinoquinazolines (compound **12**)

Mowafy *et al.* designed and synthesized a series of 4-anilinoquinazolines with C-6 ureido and thioureido side chains and various substituents at the C-4

anilino moiety was as wild type (WT) and mutant HER1 inhibitors. A few compounds from this series having bulky substitution of (3-chloro-4-(3-fluorobenzyloxy)phenyl)amino similar to that of lapatinib were also evaluated for HER2 inhibition. Compound **13** (Fig. 9) afforded most potent HER2 inhibitory activity showing IC_{50} value of 1.50/12.1 nM against HER1/HER2 kinase. The decrease in length of carbon linker between thioureido and morpholine ring from two carbons to three carbons; and the replacement of morpholine ring with piperidine ring led to decrease in HER2 inhibitory activity (Mowafy *et al.*, 2016). Ding *et al.* synthesized a series of compounds containing 4-anilinoquinazolines with C-6 triazole-linked long alkyl chains of hydroxamic acid. These compounds were evaluated for their activity against HER1, HER2 and HDAC enzymes. The selectivity between HER1 and HER2 altered upon substitution of different groups at 4-anilino position. Among these compound **14** (Fig. 9) showed the most potent HER2 inhibitory activity with IC_{50} value of 16.4 nM. It also displayed HER1 (IC_{50}=4.2 nM), HDAC1 (IC_{50}=8.3 nM) and HDAC6 (IC_{50}=19.5 nM) inhibition along with antiproliferative activity against BT-474 cells (IC_{50}=2.24μM) (Ding *et al.*, 2017).

Lin *et al.* synthesized compounds with phosphoramide mustard functionality incorporated into the quinazoline. The synthesized quinazoline derivatives were more effective against cell lines with high HER2 level (SKBR3 and H522) as compared to those with low HER2 level (HCT116 and MDA-MB-468). Compound **15** (Fig. 9) was the most potent inhibitor with IC_{50} values of 7.4/82 nM against HER1/HER2 and 2.8/2.5μM against SKBR3/H522 cells. Mechanistic studies revealed that compound **15** directly inhibited the HER1/HER2 signaling and led to the impairment of cell cycle as alkylating agent. Also, it significantly inhibited H522 tumor xenograft model showing a total growth inhibition (TGI) of 68% at dose of 100 mg/kg with no significant loss of body weight and high oral bioavailability of 72%. Further, no acute toxicity to mice at doses up to 900 mg/kg was observed. Compound **15** showed DNA damaging effects in both HCT116 and SKBR3 cells indicating the activity to be not cell-specific. SAR studies for this series of compounds indicated that increasing the length of linker between phosphoramide and quinazoline moiety from 2 to 5

carbons improved HER1/HER2 inhibition. Amide linker resulted in 3-fold decrease in potency against SKBR3 cells (Lin *et al.*, 2017).

Das *et al.* synthesized a series of quinazoline derivatives and evaluated them as HER1/HER2 inhibitors. Compounds showing significant HER1/HER2 inhibition were selected. Compounds **16** and **17** (Fig. 9) showed better inhibitory activity of HER1 (IC50 = 0.76, 0.69 nM) and HER2 (IC50 = 39.2, 42.1 nM) as compared to afatinib (HER1 IC50 = 0.96 & HER2 IC50 = 73.72nM). Whereas **18** (Fig. 9) displayed reduced HER1 inhibition (IC50 = 1.4nM) and enhanced HER2 inhibition (IC50 = 10.9nM) than afatinib. A linker of six atoms linker 7-position of quinazoline was the optimum chain length for better inhibitory activity, whereas longer or branched linkers decreased the activity. Compound **17** exhibiting good pharmacokinetic profile and oral bioavailability was selected for *in vivo* anti-tumor efficacy study. It showed better TGI rates (95.1%, 82.0%, 73.2% for high, middle and lower doses, respectively) afatinib (67.6%) in human non-small cell lung cancer cell lines NCI-H1975 mice xenograft model (Das *et al.*, 2019).

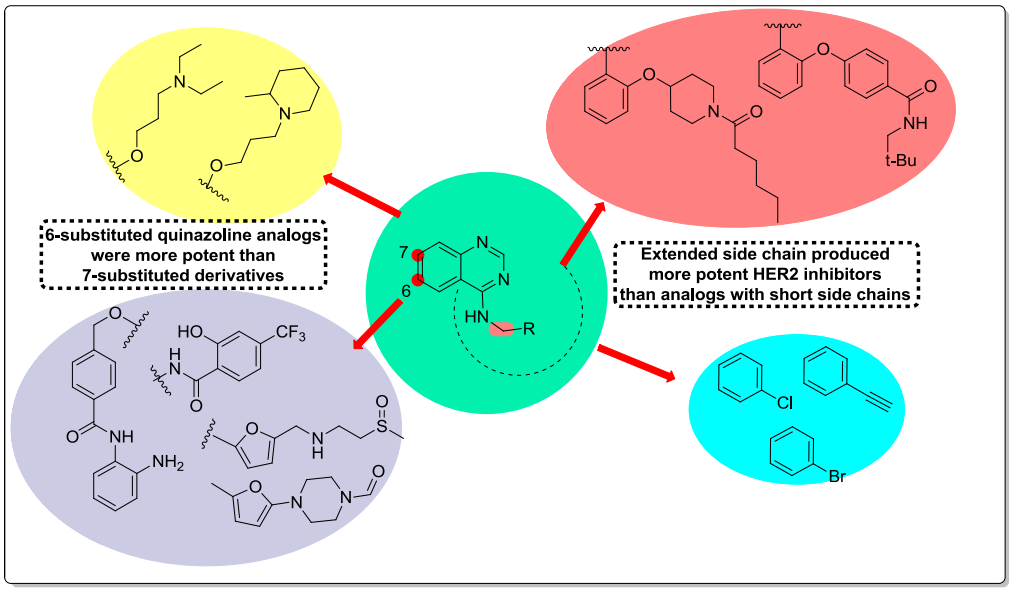


Fig. 12: SAR for various anilinoquinazoline derivatives acting as HER2 inhibitors

SAR for various anilinoquinazoline derivatives reported to be acting as HER2 inhibitors has been shown in Fig. 12.

2.16.1. Drugs under clinical investigation targeting HER2 TK

Different anilinoquinazoline-based HER2 TK inhibitors that are presently under clinical evaluation for the treatment of breast cancer have been shown in Fig. 13.

2.16.1.1. Lapatinib (GW572016/Tykerb)

Lapatinib is a reversible oral, dual TK antagonist of HER1 and HER2. It prevents the phosphorylation of their substrates and downstream signaling (Xia *et al.*, 2002; Xia *et al.*, 2005; Segovia-Mendoza *et al.*, 2015). Lapatinib displayed IC_{50} values of 10.2 nM and 8.9 nM against HER1 and HER2 TKs, respectively (Moy *et al.*, 2007). It showed activity against trastuzumab-resistant cancer cell lines. Lapatinib in combination with capecitabine was approved by FDA in March, 2007 for the treatment of metastatic breast cancer in patients who have received trastuzumab+paclitaxel and anthracycline drug regimens (Ryan *et al.*, 2008). It is found better than the trastuzumab therapy because of its activity against p95HER2 which lacks the trastuzumab-binding domain (Xia *et al.*, 2004). Lapatinib enhances sensitivity towards endocrine treatment for the HR-positive patients (Guarneri, 2009). Lapatinib in combination with letrozole, was approved by FDA in February, 2010 as a first-line therapeutic option for the treatment of post-menopausal metastatic breast cancer patients co-expressing HR and HER2 (www.accessdata.fda.gov) (Schwartzberg *et al.*, 2010). However, HER2 positive breast cancer patients develop resistance towards lapatinib monotherapy (Burris *et al.*, 2005). Resistance towards lapatinib is attributed to the HER3 transphosphorylation and activation of PI3K-Akt pathway due to negative feedback signaling loop (Sergina *et al.*, 2007) and enhanced ER signaling (Xia *et al.*, 2006). Lapatinib in combination with panobinostat+capecitabine (NCT00632489, clinicaltrials.gov) (Peacock *et al.*, 2010) and with sirolimus/metformin (NCT01087983, clinicaltrials.gov) (Aljada *et al.*, 2012) has completed phase I clinical trials for the treatment of HER2 positive metastatic breast cancer. Lapatinib along with cabazitaxel (NCT01934894, clinicaltrials.gov) has completed phase II clinical trial for treating breast cancer patients who are HER2 positive and suffering from intracranial metastases (Yardley *et al.*, 2014). The drug has completed phase II clinical trial (NCT01827163; clinicaltrials.gov) in combination with trastuzumab and paclitaxel in HER2-positive early-stage breast cancer. In combination with captecitabine, it has completed phase II clinical trial (NCT00477464; clinicaltrials.gov) for refractory, metastatic HER2-positive breast cancer.

Lapatinib is currently undergoing phase II clinical trial (NCT00820872; clinicaltrials.gov)(Iyengar *et al.*, 2014) and phase III trial (NCT00553358, clinicaltrials.gov) (José Baselga *et al.*, 2012; De Azambuja *et al.*, 2014) in various combinations of trastuzumab and other chemotherapeutic agents for the treatment of HER2 positive early stage breast cancer and as neoadjuvant therapy for the HER2-positive breast cancer. Apart from this, lapatinib is undergoing various other clinical studies for the treatment of breast cancer and other solid tumors (www.clinicaltrials.gov).

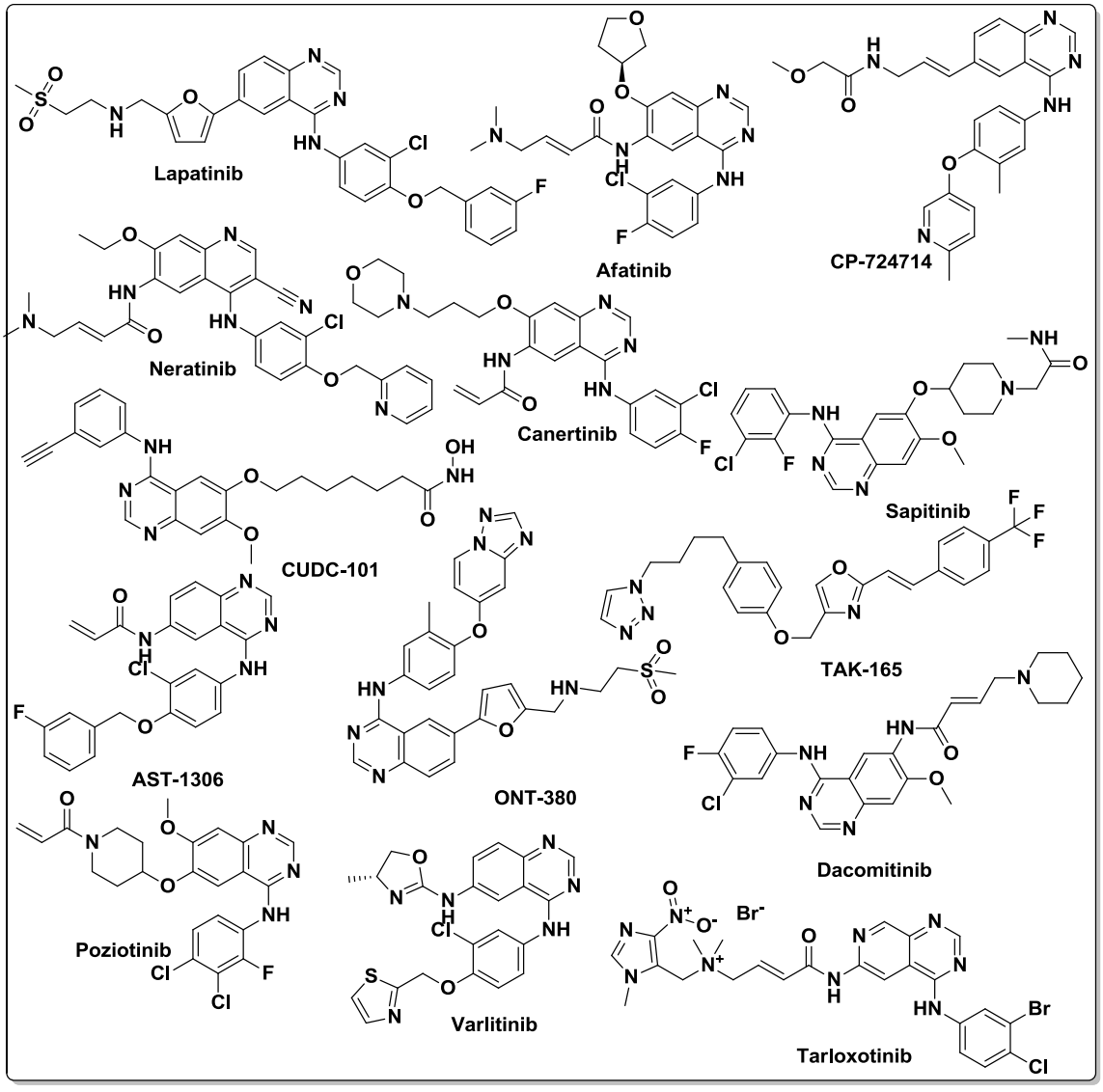


Fig. 13: Structures of anilinoquinazoline-based HER2 inhibitors undergoing clinical trials

2.16.1.2. Neratinib (HKI-272)

On July 17, 2017, neratinib has been approved by FDA for the extended adjuvant treatment of adult patients with early-stage HER2-positive breast

cancer, post adjuvant trastuzumab-based therapy (Deeks, 2017). Neratinib is an oral, irreversible inhibitor of HER1/HER2 with IC_{50} value of 92/59 nM (Rabindran *et al.*, 2004; Rabindran, 2005; Tsou *et al.*, 2005; Burstein *et al.*, 2010). In phase III clinical evaluation (NCT00878709, clinicaltrials.gov), neratinib has shown improved invasive disease-free survival in HER2 positive early breast cancer patients who have previously obtained adjuvant trastuzumab therapy (Martin *et al.*, 2017). The drug in combination with capecitabine and vinorelbine, respectively (NCT00741260 and NCT00706030, clinicaltrials.gov) has shown promising antitumor activity in HER2 positive metastatic breast cancer patients and the toxicity profile was also manageable (Awada *et al.*, 2012; Saura *et al.*, 2014). Relevant clinical activity and acceptable tolerability for neratinib as monotherapeutic agent (NCT00777101, clinicaltrials.gov) in HER2 positive recurrent or metastatic breast cancer has been observed. Moreover, neratinib can also be used as an alternative therapy for patients who do not respond to therapeutic combination of lapatinib and capecitabine (Martin *et al.*, 2013).

In a phase II study (NCT00915018, clinicaltrials.gov) relative to a combination of trastuzumab and paclitaxel, the combination of neratinib plus paclitaxel has been found to be more effective in reducing CNS progression associated with HER2 positive breast cancer (Awada *et al.*, 2015). HER2 activating mutations have been found in HER2 negative tumors. Although these tumors lack HER2 gene amplification, these have been found to benefit from HER2 targeted agents. Neratinib as single agent has shown marked improvement in a young woman suffering metastatic breast cancer and carrying HER2 LSS5 mutation in HER2 tyrosine kinase domain (Ben-Baruch *et al.*, 2015).

2.16.1.3. Afatinib

Oral afatinib (BIBW 2992 / Giotrif / Gilotrif; Boehringer Ingelheim) is an approved first-line treatment for HER1-positive metastatic non-small cell lung carcinoma (NSCLC) patients, possessing HER1 exon 19 deletions or exon 21 (L858R) substitution mutations (www.accessdata.fda.gov). Afatinib is orally bioavailable pan-HER inhibitor targeting HER1, HER2 and HER4 via irreversible covalent binding interactions. It also inhibits HER3 transphosphorylation (Hirsh, 2011). Afatinib exhibits covalent interactions with Cys 797 of HER1, Cys 805 in HER2 and Cys 803 in HER4 (Geuna *et al.*, 2012; Solca *et al.*, 2012). It showed half-

maximal inhibitory concentration values of 0.5 nM, 14 nM and 1 nM against HER1, HER2 and HER4, respectively (Geuna *et al.*, 2012; Solca *et al.*, 2012). The drug retains in vivo and in vitro anticancer activity in tumors that acquire resistance towards reversible HER1 inhibitors due to T790M mutation (Regales *et al.*, 2009; Takezawa *et al.*, 2010; Kim *et al.*, 2012; Tabara *et al.*, 2012; Lee *et al.*, 2013; Mack *et al.*, 2013; Ninomiya *et al.*, 2013).

Although, afatinib is an effective first-line treatment for NSCLC but the drug is still under clinical evaluation for the treatment of HER2 positive breast cancer (clinicaltrials.gov). Afatinib and/or vinolerobine has completed phase II clinical trials (NCT01441596 and NCT01325428, clinicaltrials.gov) for the treatment of HER2 overexpressing metastatic breast cancer patients with extracranial brain metastases and HER2 overexpressing inflammatory breast cancer (Joensuu *et al.*, 2012; Swanton *et al.*, 2012). Afatinib has successfully completed phase II trial (NCT00431067, clinicaltrials.gov) for the treatment of trastuzumab-refractory HER2 positive metastatic breast cancer. Promising clinical activity was observed in this study. The toxicity profile was also manageable, however, frequent diarrhea was observed with afatinib monotherapy (Lin *et al.*, 2012).

Afatinib has been found to be quite effective over trastuzumab and lapatinib (NCT00826277, clinicaltrials.gov) in the neoadjuvant treatment of HER2 positive breast cancer (Rimawi *et al.*, 2015). Afatinib alone or in combination with vinolerobine has completed phase II clinical evaluation (NCT01271725, clinicaltrials.gov) for treating HER2 positive breast cancer patients who were not responding to HER2 targeted therapy in adjuvant/neoadjuvant settings showing promising activity (Hickish *et al.*, 2012; Lin *et al.*, 2012). In combination with trastuzumab, the drug has completed phase I clinical evaluations (NCT01649271, NCT00950742; clinicaltrials.gov) for treating HER2 overexpressing breast cancer. Afatinib also completed phase III clinical trial (NCT01125566, clinicaltrials.gov) for evaluation against metastatic HER2-positive breast cancer. However, when a comparative analysis for afatinib plus vinolerobine versus trastuzumab plus vinolerobine treatments was done, trastuzumab-based therapy remained as treatment of choice for those metastatic

patients who progressed on previous trastuzumab-therapy (Harbeck *et al.*, 2016).

2.16.1.4. Sapitinib (AZD8931)

Sapitinib (AZD8931) is a reversible equipotent inhibitor of HER1, HER2 and HER3-mediated signaling dynamics displaying IC_{50} values of 4 nM, 3 nM and 4 nM respectively (Hickinson *et al.*, 2010; Barlaam *et al.*, 2013). Methyl acetamide chain of sapitinib contributes to the increased HER2 activity. It exhibited potent tumor growth inhibition in various xenograft models either expressing HER1 or co-expressing HER1 and HER2 (Barlaam *et al.*, 2013). Sapitinib in combination with paclitaxel has successfully completed phase II clinical trials (NCT00900627, clinicaltrials.gov) for treating female subjects suffering from advanced breast cancer with low HER2 overexpression (Baselga *et al.*, 2013).

2.16.1.5. CP-724, 714

CP-724,714 is a reversible, potent and selective oral HER2 TK inhibitor exhibiting IC_{50} value of 10 nM. It induced cell-cycle arrest at G_1 phase in BT474 carcinoma cell lines. Treatment with CP-724,714 decreases HER2 receptor phosphorylation in *ex vivo* studies (Jani *et al.*, 2007). It was found to foster cell apoptosis and reduction in downstream receptor TK signaling. It caused tumor-regression in HER2 overexpressing tumor cell lines (Jani *et al.*, 2007). The drug inhibited hepatic efflux transporters that caused hepatic accumulation of drug and bile constituents leading to hepatocellular injury and hepatobiliary cholestasis (Feng *et al.*, 2009). CP-724,714 has completed phase I clinical trial (NCT00055926; clinicaltrials.gov) for the treatment of metastatic HER2-positive breast cancer.

2.16.1.6. Canertinib (CI-1033)

Canertinib (CI-1033) is an oral, irreversible pan-HER TK inhibitor, obstructing signaling from all members of the EGFR family i.e., HER1, HER2, HER3 and HER4 (Slichenmyer *et al.*, 2001). It showed IC_{50} values of 1.5/9.0 nM against HER1/HER2 (Smaill *et al.*, 2001). In a randomized phase II trial, the drug exhibited no significant clinical activity as a monotherapeutic agent in heavily pre-treated metastatic breast cancer patients. However, antitumor activity with enhanced overall survival rate was observed in one arm of HER2 positive

patients at a drug dosage of 50 mg. Toxicity was observed at higher doses (Rixe *et al.*, 2009).

2.16.1.7. CUDC-101

CUDC-101 is an irreversible, multi-targeted inhibitor of HDAC, HER1, and HER2 with IC_{50} values of 4.4 nM, 2.4 nM, and 15.7 nM, respectively (Cai *et al.*, 2010). In various xenograft models, the drug has shown tumor regression. In addition, CUDC-101 also exhibited inhibition of other alternate signaling pathways such as Akt, HER3, MET and hence, conquering the restraints of conventional HER1/HER2 inhibitors (Lai *et al.*, 2010). It has successful completed phase I clinical trial (NCT01171924, clinicaltrials.gov) in advanced head and neck, gastric, breast, liver and non-small cell lung cancer. The drug is well-tolerated along with possession of antitumor activity (Shimizu *et al.*, 2014).

2.16.1.8. AST-1306

AST-1306 is an oral and irreversible inhibitor of HER1/HER2 with IC_{50} values of 0.5/3.0 nM. It binds through covalent interaction with Cys797 and Cys805 in the catalytic domains of HER1 and HER2, respectively. HER2 overexpressing cell lines, tumor xenografts and breast cancer mouse models showed more sensitivity towards the drug as compared to HER1 overexpressing cell lines (Xie *et al.*, 2011). AST-1306 completed phase I open-label dose-escalation study to determine its safety and tolerability, pharmacokinetics and preliminary anti-tumor effects which indicated rapid absorbance of the drug with moderate to high clearance. The maximum tolerated dose of the drug was found to be 1000 mg and it was further recommended for additional phase II trials (Zhang *et al.*, 2014).

2.16.1.9. Dacomitinib (PF00299804)

Dacomitinib (PF00299804) is an oral irreversible pan-HER inhibitor of HER1, HER2, and HER4 with IC_{50} values of 6.0 nM, 45.7 nM, and 73.7 nM, respectively. The drug inhibited wild-type HER2 and the gefitinib-resistant oncogenic HER2 mutation in lung cancers (Engelman *et al.*, 2007). Dacomitinib exhibited significant antitumor activity in tumor xenograft models expressing and/or overexpressing HER family members or having mutations in HER1 (Gonzales *et al.*, 2008). Dacomitinib caused G_0-G_1 arrest and apoptosis induction in order to exert its antiproliferative activity. An escalating growth inhibitory activity was observed for HER2-amplified cell lines which was maintained by the drug in the

corresponding cell lines showing resistance against trastuzumab and lapatinib (Kalous *et al.*, 2012). Dacomitinib, as monotherapeutic agent has completed an open-label phase II trial (NCT01152853, clinicaltrials.gov) in HER2 positive advanced gastric cancer patients who have prior failed at least one chemotherapy regimen (Oh *et al.*, 2012).

2.16.1.10. ONT-380 (ARRY-380 or tucatinib)

ONT-380 (ARRY-380 or tucatinib) is a small-molecular TK inhibitor of HER2 with an IC_{50} value of 8 nM. It was found to be 500 times more selective for HER2 as compared to HER1 (Borges *et al.*, 2014). The drug in combination with trastuzumab and ado-trastuzumab emtansine is undergoing phase I clinical trials (NCT01921335 and NCT01983501, respectively; clinicaltrials.gov) for the treatment for HER2 positive advanced breast cancer (Borges *et al.*, 2014; Metzger-Filho *et al.*, 2014). It is also under clinical phase I evaluation in combination with capecitabine and/or trastuzumab for HER2-positive breast cancer (NCT02025192, clinicaltrials.gov). ONT-380 combined with palbociclib and letrozole is under phase I clinical trial, for the treatment of luminal/HER2-positive breast cancer (NCT03054363, clinicaltrials.gov).

2.16.1.11. Poziotinib (HM781-36B; NOV120101)

Poziotinib is an irreversible pan-HER inhibitor with IC_{50} values of 3 nM, 5 nM and 23 nM for HER1, HER2, and HER4 respectively. It has shown anti-proliferative effects against both HER1 and HER2 overexpressing cell-lines and tumor xenografts. Besides, it exhibited an IC_{50} value of 1.0 nM against HER2 overexpressing SKBR3 cell lines (Cha *et al.*, 2012). Poziotonib stimulated cell cycle arrest at G_1-phase and apoptosis, and reduces the levels of HER family and downstream signaling molecules (Kang *et al.*, 2015). It displayed potent *in vitro* and *in vivo* antitumor activity against HER2 amplified gastric cancer cells (SNU216 and N87). In combination with chemotherapeutic agents, synergistic effects of poziotinib were seen in both HER2 amplified and HER2 non-amplified gastric cancer cells (Nam *et al.*, 2011).

In metastatic HER2 positive breast cancer patients**,** drug acclaims promising clinical activity (Im *et al.*, 2016). Poziotinib entered phase II evaluation (NCT02418689, clinicaltrials.gov) in patients with HER2-positive metastatic breast cancer who have received at least two prior HER2-directed regimens (Y.

Park *et al.*, 2016). At present, the drug is undergoing phase II evaluation for treating metastatic HER2-positive breast cancer (NCT02659514, clinicaltrials.gov) and phase I evaluation in combination with ado-trastuzumab emtansine for HER2-positive breast cancer (NCT03429101).

2.16.1.12. Varlitinib (ARRY-334543; ASLAN001)

Varlitinib is a potent, reversible, tyrosine kinase inhibitor of HER1, HER2 and HER4 showing IC_{50} values of 7 nM, 2 nM and 0.195 nM, respectively. It has completed phase II study (NCT01614522,clinicaltrials.gov) in patients with recurrent/metastatic gastric cancer whose tumors are either HER2 amplified or co-expressing HER1 and HER2 (Kim *et al.*, 2014).

2.16.1.13. Tarloxotinib (TH-4000)

Tarloxotinib bromide is a prodrug activated under hypoxic conditions to release an irreversible HER1/HER2 inhibitor. It entered phase II clinical investigation (NCT02454842, clinicaltrials.gov) for treating HER1 mutant T790M-negative NSCLC subjects who have progressed on HER1-TKI (Silva *et al.*, 2015).

CHAPTER 3

MATERIAL AND METHODS

3.1. Patient recruitment

The study group consisted of female HER2-positive breast cancer patients. These were recruited from the oncology departments of Guru Gobind Singh Medical College and Hospital (GGSMCH), Faridkot and Max Super-Speciality Hospital, Bathinda, Punjab. Patients confirmed to be affected by breast cancer were further evaluated for HER2 overexpression by IHC. As per the IHC evaluation, there were 90 confirmed HER2 (3+) and 19 HER2 (2+)/equivocal cases those were evaluated in between September, 2015 and February, 2017.

3.2. FISH evaluation

The HER2 (2+)/equivocal cases (n=19) were evaluated using fluorescence *in situ* hybridization (FISH) technique to confirm the HER2-positive status. FISH is a molecular cytogenetic technique that facilitates the identification of genomic aberrations such as gene amplifications, deletions and chromosomal rearrangements in metaphase and interphase cells. In cancer cells, this technique is used for the assessment of HER2 amplification. We used PathVysion Kit (Abbott Laboratories, Abbott Park, IL, USA) for FISH evaluation. PathVysion kit uses dual probes for determining the number of copies of HER2/neu gene and the centromeric region of chromosome 17.

Principle

FISH works on the principle of hybridisation of fluorescent-labelled probes to the complementary DNA target sequences. Before use, PathVysion probes are mixed and denatured in hybridisation buffer. FFPE tissue specimens are placed on slides and DNA is denatured into single-stranded form. Denatured DNA is allowed to hybridise with the PathVysion Probes. The SpectrumOrange, Locus Specific Identifier (LSI) probe (226) Kb targets HER2/neu gene locus (17q11.2-q12) and the SpectrumGreen, Chromosome Enumeration Probe 17 (CEP17) targets α-satellite DNA sequence at the centromeric region of chromosome 17 (17p11.1-q11.1). Following hybridisation, the intense orange (HER2/neu) and green (CEP17) fluorescent signals are visualised using fluorescence microscope. The ratio of HER2/neu gene to chromosome 17 copy number is calculated by the enumeration of HER2/neu and CEP17 signals.

Materials required

1. LSI HER-2/neu SpectrumOrange (low copy number E. coli vector)/ CEP 17 SpectrumGreen DNA Probe (E. coli plasmid): Pre-denatured in hybridisation buffer.
2. DAPI Counterstain: 1000 ng/mL DAPI (4,6-diamidino-2-phenylindole) in phenylenediamine dihydrochloride, glycerol, and buffer.
3. NP-40: Igepal (NP-40 substitute) [Octyl phenoxy]polyethoxyethanol.
4. 20X SSC (sodim chloride and sodium citrate) salts
5. Vysis Paraffin Pretreatment Reagent Kit that includes Vysis Pretreatment Solution (NaSCN), Vysis Protease(Pepsin), Vysis Protease Buffer (NaCl solution, pH 2.0) and Vysis Wash Buffer (2X SSC, pH 7.0)
6. Neutral buffered formalin solution (4% formaldehyde in PBS)
7. Hemo-De clearing agent
8. Hematoxalin and eosin
9. Immersion oil appropriate for fluorescence microscopy
10. Ultra-pure, formamide
11. Ethanol (100%)
12. Concentrated (12N) HCl
13. 1N NaOH
14. Purified water (distilled or deionized or Milli-Q)
15. Rubber cement
16. Drierite
17. ProbeChek HER-2/neu Normal Control Slides (Normal Signal Ratio): FFPE cultured human breast cancer cell line (MDA-MB-231; normal LSI HER-2/neu:CEP 17 ratio) applied to glass microscope slides.
18. ProbeChek HER-2/neu Cutoff Control Slides (Weakly Amplified Signal Ratio): FFPE cultured human breast cancer cell line (Hs 578T; low level HER-2/ neu amplification) applied to glass microscope slides.

    Note: Control slides must be stored at 15 to 30°C in a sealed container with desiccant to protect them from humidity.

    Preparation of working reagents

    1. 20X SSC (3M sodium chloride, 0.3M sodium citrate, pH 5.3): Mix 66 g of 20X SSC and 200 ml of purified water thoroughly. Adjust pH to 5.3

with concentrated HCl. Add purified water to bring the total volume upto 250 ml. The 20X SSC solution can be stored at room temperature upto 6 months.

2. Denaturing Solution (70% formamide/2X SSC, pH 7.0-8.0): Add 49 ml of formamide and 7 ml of 20X SSC in 14 ml of purified water. Store this solution at 2 to 8°C in air-tight container. It can be used for up to 7 days.
3. Ethanol Solutions: Prepare v/v dilutions of 70%, 85%, and 100%, using 100% ethanol and purified water. Store these ethanolic dilutions at room temperature in air-tight containers. Dilutions can be used upto1 week keeping check on evaporation or further dilution of the solution due to excessive use.
4. Post-Hybridization Wash Buffer (2X SSC/0.3% NP-40): Add together 100 Ml of 20X SSC, 847 ml of purified water and 3 ml of NP-40 and mix thoroughly. Using 1N NaOH, adjust pH to 7.0-7.5. Make final volume upto 1000 ml with purified water. At the end of each day, the used solution must be discarded. The unused solution must be stored at room temperature for up to 6 months.

Procedure

i. **Slide preparation from FFPE tissue:** Using a microtome cut 4-6 µm thick paraffin sections make these sections float in a protein-free water bath at 40±2°C. Tissue section must be mounted on the positive side of an organosilane-coated slide. Then, slides are allowed to air-dry and baked overnight at 56°C.

ii. **Slide-pretreatment:** Steps for slide-pretreatment are as follows:

- *Deparaffinizing slides:* Slides are immersed in Hemo-De for 10 minutes at room temperature. Repeat the slide immersion in Hemo De twice using new Hemo-De each time. Then, slides are dehydrated in 100% ethanol for 5 minutes at room temperature. Dehydration in 100% ethanol is repeated again. Slides are air-dried or are placed on a 45-50°C slide warmer.
- *Pretreating slides:* Slides are immersed in 0.2N HCl for 20 minutes, followed by immersion in purified water and wash buffer for 3 minutes, each. Slides are then placed in Pretreatment Solution at 80±1°C for 30

minutes. After that, slides are immersed in purified water for 1 minute. Subsequently, slides are placed in wash buffer for 5 minutes. Immersion in wash buffer is repeated again.

- *Protease treatment:* Excess buffer is removed by blotting edges of the slides on a paper towel. Slides are immersed in Protease Solution at 37±1°C for 10 to 60 minutes and then, in Wash Buffer for 5 minutes. Immersion in wash buffer is repeated again. Slides are dried on a 45-50°C slide warmer for 2-5 minutes.
- *Fixing the specimen:* Slides are immersed in neutral buffered formalin at room temperature for 10 minutes and then, in wash buffer for 5 minutes. Immersion in wash buffer is repeated again. Slides are dried on a 45-50°C slide warmer for 2-5 minutes, before proceeding with the PathVysion assay protocol.

iii. Probe preparation: Probe is allowed to warm to room temperature and properly mixed using vortex. Each tube is centrifuged for 2-3 seconds and contents are allowed to mix gently employing vortex.

iv. Denaturation of Specimen DNA: DNA is denatured as per the following steps:

- Prior to slide preparation, the humidified hybridization chamber (an airtight container with a piece of damp blotting paper approximately 1 in×3 in taped to the side of the container) is pre-warmed to 37±1°C by placing in incubator. Blotting paper is moistened with water before each use of the hybridization chamber.
- pH of the denaturing solution should be carefully checked before use. It should be 7.0-8.0 at room temperature. Denaturing solution is added to Coplin jar and placed in a 72±1°C water bath for at least 30 minutes.
- The areas to be hybridized are marked using a diamond-tipped scribe.
- The prepared slides are immersed in the denaturing solution at 72±1°C (≤6 slides per jar) for 5 minutes to denature the specimen DNA.
- Slides are removed from the denaturing solution and immediately placed into a 70% ethanol wash solution at room temperature. Slide are agitated to remove the formamide. Slides are allowed to stand in the ethanol wash for 1 minute.

- Slides are removed from 70% ethanol. Step 5 is repeated with 85% ethanol, followed by 100% ethanol.
- Excess ethanol is drained from the slide by touching the bottom edge of the slide to a blotter and wiping the underside of the slide dry.
- Slides are dried on a 45-50°C slide warmer for 2-5 minutes.

Note: There should be coordination between the timing for preparing the probe solutions and denaturing the specimen DNA so that both should be ready for the hybridization step at the same time.

v. Hybridization: Hybridization is done as follows:

- 10 µL of probe mixture is applied to the target area of slide. A 22 mm×22 mm glass coverslip is immediately placed over the probe and probe is allowed to spread evenly under the coverslip. Air buSbbles should be avoided, as these can interfere with hybridisation. After use, the remaining probe solution should be refrozen immediately
- The rubber cement is drawn into a 5 mL syringe. A small amount of rubber cement is ejected around the periphery of the coverslip overlapping the coverslip and the slide, such that a seal is formed around the coverslip.
- Slides are placed in the prewarmed humidified hybridization chamber. The chamber is covered with a tight lid and incubated at 37±1°C overnight (14-18 hours), before proceeding with the post-hybridization washes.

vi. Post-hybridization Washes: Post-hybridization steps are preformed in accordance with steps below:

- Post-hybridization wash buffer (2X SSC/0.3% NP-40) is added to a Coplin jar. Buffer is pre-warmed by placing the Coplin jar in the 72±1°C water bath for at least 30 minutes.
- Post-hybridization wash buffer is added to a second Coplin jar and placed at room temperature. Both wash solutions should be discarded after 1 day of use.
- The rubber cement seal from the first slide is removed by gently pulling up on the sealant with forceps.
- Slides are immersed in post-hybridization wash buffer at room temperature.

- Coverslip is carefully removed, then excess liquid is removed by wicking off the edge of the slides and slides are immersed in post-hybridization wash buffer at 72±1°C for 2 minutes (6 slides/jar).
- Each slide is removed from the wash bath and air-dried in the dark (a closed drawer or a shelf inside a closed cabinet) in an upright position.
- 10 µL of DAPI counterstain is applied to the target area of the slide and a glass coverslip is applied over that. Slides are stored at –20°C in the dark prior to signal enumeration. Slides must be allowed to reach room temperature prior to viewing under fluorescence microscope.

vii. **Signal Enumeration:** A 25X objective is used to view the hybridized area. Tumor cells are identified by H & E stain. Several areas of tumor cells are scanned to account for possible heterogeneity using a 40X objective. An area of good nuclei distribution is selected. Areas of the target where hybridization signals are weak and non-specific or with noisy background; should be avoided. Upper left quadrant of the selected area is analysed using a 63X or 100X objective. The number of signals within the nuclear boundary of each evaluable interphase cell is counted scanning from left to right. Target area should be focussed up and down to find all of the signals present in the nucleus.

The number of LSI HER-2/neu signals and the number of CEP 17 signals are counted for each nucleus. 2 signals of the same size and separated by a distance equal or less than the diameter of the signal are counted as 1 signal. Nuclei with no signals or with signals of only 1 color are not scored. Only those nuclei with 1 or more FISH signals of each color are scored. To view both color signals, alternate between the DAPI/9-orange, DAPI/green, Green/Orange (V.2), and the DAPI/ green/Orange (V.2) filter sets.

viii. **Determination of LSI HER-2 to CEP 17 Ratio:** LSI HER-2/neu to CEP 17 ratio is calculated by dividing the total number of LSI HER-2/neu signals by the total number of CEP 17 signals in counting the same 20 nuclei. The number of LSI HER-2/neu and CEP 17 counts in 20 nuclei is recorded. All LSI HER-2/neu signals are added to get total LSI HER-2/neu signals and all CEP 17 signals are added to get the total CEP 17 signals. If the ratio of

total LSI HER-2/neu signals to total CEP 17 signals is <2, theHER-2/neu gene amplification was not observed. If the ratio is ≥2, HER-2/neu gene amplification was observed.

Note: Count an additional 20 nuclei, if the LSI HER-2/neu to CEP 17 ratio is borderline (1.8 to 2.2), and recalculate the ratio based on the total of 40 nuclei.

ix. Quality Control

To monitor assay performance and to assess the accuracy of signal enumeration, control slides are run concurrently with patient slides

3.3. Exclusion criteria: Breast cancer patients with negative HER2 status and patients with major cardiac, renal, hepatic, skeletal disorders, other cancers or neurological disorders were excluded from this study.

3.4. Inclusion criteria: Breast cancer patients confirmed to be positive for HER2 status were only included in the study.

3.5. Control recruitment

100 age-matched healthy female volunteers belonging to same demographic area were recruited as a control group from the same demographic area. The controls had no personal/familial history of breast cancer or any other cancer.

3.6. Data Collection

Information on demographic features including age (at the time of diagnosis, menarche and first pregnancy), height, weight, residential area, menopausal status, history of hysterectomy, family history of breast cancer/other cancers and pesticide exposure was collected for all the subjects included in the study. This information was recorded in structured questionnaires. Written informed consent for sample collection was obtained from all the HER2-positive breast cancer patients (n=100 including 90 IHC-3+ and 10 FISH+ cases) and controls (n=100). The approval for this study was acquired from the institutional ethics committees of the Central University of Punjab.

3.7. Follow-up

Trastuzumab treatment was given to 70% (70/100) of the patients. Follow-up of these patients was done telephonically and with the help of clinician during their visits to the hospitals at the interval of 3, 6, 9, 12, 15, 18, 21, 24 and 27 months from the last administered cycle of trastuzumab. Median follow-up was 13.5 months.

Details about metastasis, recurrence and death among these patients were collected with the help of clinician as well as through telephonic interview of the patients. Based on death, metastasis recurrence, the patients were classified into patients with good and bad outcome.

3.8. Sample Collection

5 ml of blood was collected in vacutainers containing ethylenediaminetetraacetic acid (EDTA) as an anti-coagulant from all HER2+ BC patients (n=100) and controls (n=100). Blood samples collected from the patients and healthy controls are stored at -20^{0}C.

Formalin-fixed paraffin-embedded (FFPE) tissue samples could be collected only for 20 HER2-positive breast cancer patients to whom trastuzumab treatment was given. However, we weren't able to collect matched tissue samples from healthy volunteers on account of ethical concerns.

3.9. DNA isolation by Phenol chloroform method

Principle

The aqueous solution of nucleic acids is often extracted with Phenol:Chloroform:Isoamylalcohol to facilitate the removal of proteins. Before extraction with the organic solvents, most of the protein content is removed by digestion with Proteinase K. Proteinase K digests the cellular proteins leaving behind the crude lysate. Further, addition of SDS causes breakage of disulphide bonds.

Phenol brings about protein denaturation, while Chloroform separates the aqueous phase and organic phase. Foaming during extraction is minimized using Isoamylalcohol. Ethanol aids in DNA precipitation and removal of the remaining salts.

Preparation of Reagents

1. **Lysis Buffer I (LB I- 10X, 1 liter, pH=8.0)**
    a. Ammonium chloride: 83 g
    b. Potassium carbonate: 10 g
    c. 0.5 M EDTA: 2 ml

    Dissolve the contents in 800 ml of distilled water and make the volume upto 1000 ml. Store the stock solution (10X) at 4^{0}C. Working solution (1X) is prepared freshly for each use.

2. **Lysis Buffer II (LB II- 1X, 100 ml, pH=8.0)**
    a. 1 M Tris: 500 µl
    b. Sodium chloride: 1.168 g
    c. 0.5 M EDTA: 200 µl

Dissolve the contents in 80 ml of distilled water and make the volume upto 100 ml. Store the solution (1X) at 4^0C. Buffers can be used until these are clear with no precipitation.

3. **1 M Tris (50 ml, pH=8.0)**

Dissolve 6.05 g of Tris base in 40 ml of distilled water and make the volume upto 50 ml. Adjust the pH to 8.0 with the help of concentrated HCl.

4. **0.5 M EDTA (50 ml, pH=8.0)**

Dissolve 9.305 g of EDTA (disodium salt) in 40 ml of distilled water and make the volume upto 50 ml. Adjust the pH to 8.0 with the help of NaOH pellets.

5. **10% SDS:**

Dissolve 10 g of sodium dodecyl sulphate (anionic detergent) in 50 ml of distilled water. Stir slowly to avoid foaming. Make up the volume to 100 ml, after the foam settles down. Incubate at 55^0C for complete dissolution. 10% SDS can be stored at room temperature.

6. **Proteinase K**

Dissolve 20 mg of proteinase K in 1ml of distilled water. Solubilisation takes around 30 minutes. Store it at -20^0C.

7. **Tris-equilibrated phenol**

It is a phenol solution (pre-equilibrated with 10 mM Tris HCl, pH 8.0, with 1 mM EDTA) available with Merck. A layer of Tris buffer caps the clear phenol solution. Tris-equilibrated phenol is stored at 4^0C. The equilibration of phenol to pH 8.0, is must to avoid the partitioning of DNA into organic phase at acidic pH.

8. **Chloroform**
9. **Isoamylalcohol**
10. **Sodium acetate (3M, pH=5.2)**

It is supplied by Thermo-Fischer Scientific and can be stored at room temperature.

11. Absolute Ethanol

Chilled ethanol is used for DNA precipitation.

12. 70% Ethanol

Take 70 ml of ethanol and make the volume upto 100 ml with the help of distilled water.

13. T.E. buffer (pH=8.0)

1 ml of 1 M Tris (pH=8.0) and 200 µl of 0.5 M EDTA (pH=8.0) are mixed and a final volume of 100 ml is prepared with the help of distilled water. The buffer is stored at room temperature.

14. Xylene

Xylene is used for deparaffinization of FFPE tissue samples, prior to DNA extraction.

Procedure

DNA isolation from blood samples

Frozen blood was thawed and allowed to come at room temperature. To 2 ml of whole blood taken in centrifuge tube, 6 ml of lysis buffer-I (1X) was added and kept in ice for 15 minutes. The tube was removed from ice, shaken vigorously and centrifuged at 3,000 rpm for 15 minutes at 4^0C. A pellet was formed at the bottom of tube and the supernatant was discarded. Again, the addition of 6 ml of lysis buffer-I (1X) was made and the tube was kept in ice for 15 minutes. After 15 minutes, tube was recovered from ice bath, shaken vigorously and centrifuged at 3,000 rpm for 15 minutes at 4^0C. Supernatant was discarded, leaving the pellet behind. The process was repeated for 1-2 times, until a white pellet was obtained. Vortex was done and; 2 ml of lysis buffer-II (1X), 112 µl of 10% SDS and 50 µl of proteinase K were added. Sample was placed in water bath at 37^0C for overnight incubation. Next day, sample was recovered from water bath and allowed to come at room temperature.

An equal volume of phenol:chloroform:isoamylalcohol in the ratio of 25:24:1 was added to this followed by gentle mixing for 15-20 minutes till emulsion formation took place. The emulsion was centrifuged at 2,500 rpm for 15 minutes. The aqueous layer was transferred to fresh tube with the help of wide-bore tip. To this, an equal volume of chloroform:isoamylalcohol in the

ratio of 24:1 was added, mixed gently for 15-20 minutes and centrifuged at 2,500 rpm for 15 minutes.

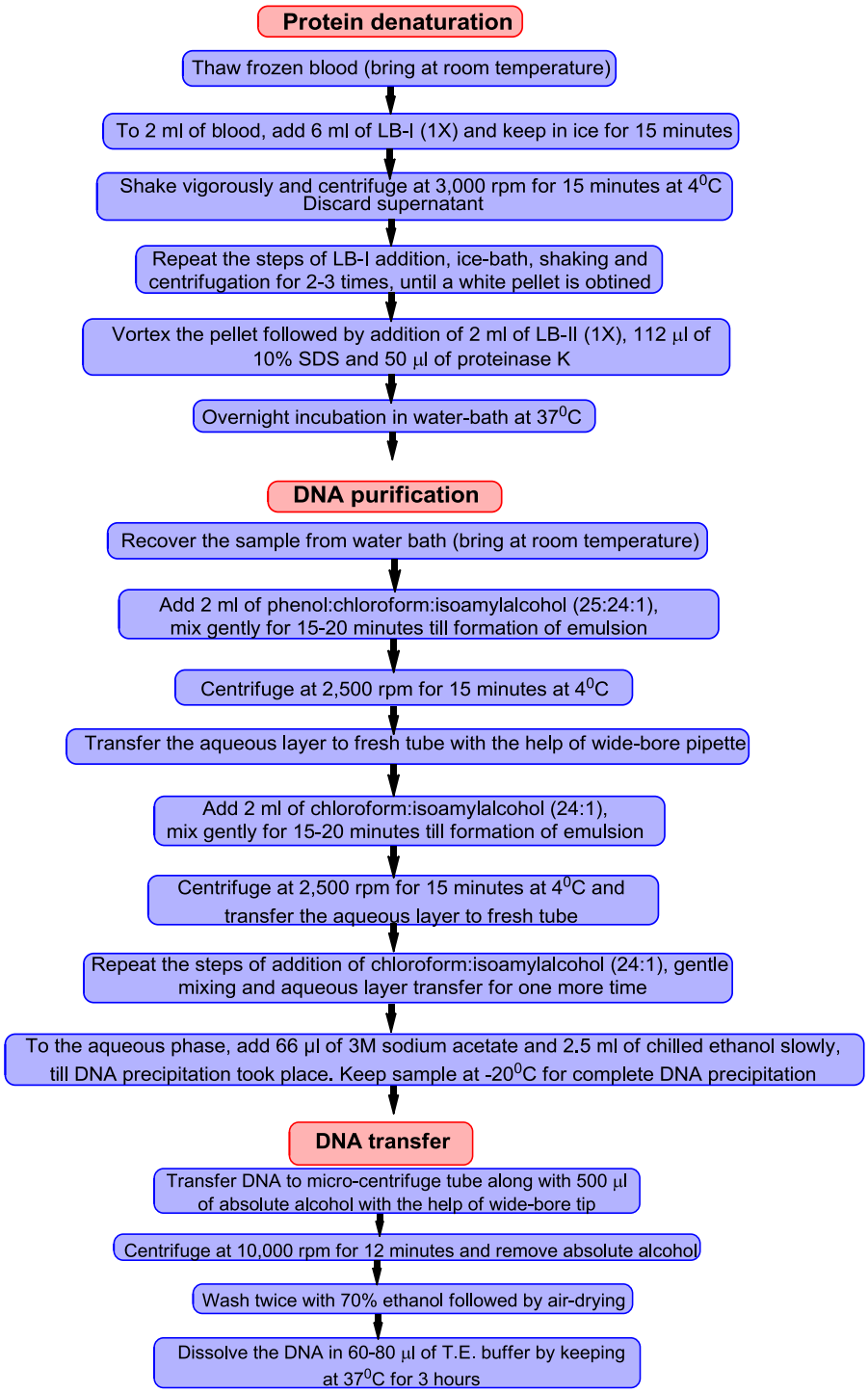


Fig. 14: DNA isolation procedure from blood samples

With the help of wide-bore tip, aqueous layer was transferred to fresh tube followed by addition of chloroform:isoamylalcohol (24:1) in equal volume, gentle mixing 15-20 minutes and centrifugation at 2,500 rpm for 15 minutes.

To the aqueous phase, 66 µl of 3M sodium acetate and 2.5 ml of chilled ethanol was added and mixed slowly, till DNA precipitation took place. Sample was kept at -20^{0}C for complete DNA precipitation. DNA along with 500 µl of absolute alcohol was transferred to the eppendorf with the help of wide-bore tip. Then, DNA was centrifuged at 10,000 rpm for 12 minutes. Absolute alcohol was removed from it and washed twice with 70% ethanol followed by air-drying to remove traces of alcohol. DNA was dissolved in 60-80 µl of T.E. buffer by keeping at 37^{0}C for 3 hours. For prolonged storage, isolated DNA samples were kept at -20^{0}C (Fig. 14).

DNA isolation from FFPE tissue

1. FFPE Tissue sample pre-processing: Deparaffinization

Following are the steps for deparaffinization of FFPE tissues:

- 1 ml of xylene is added to the tissue core taken in micro-centrifuge tube and vortexed vigorously for 10 sec. This is followed by heating for 3 min at 50 °C.
- Tube containing tissue is put on centriguration for 2 min at 10,000 rpm and then, placed in ice for 5 min. This makes the waxy residue to solidify around the meniscus of tube.
- Paraffin along with supernatant is carefully with the help of a pipette tip.
- Steps 1 and 2 are repeated followed by addition of 1 ml of 100% ethanol and vigorous mixing using vortex for 10 sec. Centrifugation is done for 2 min at 10,000 rpm and ethanol is discarded, carefully. Step 3 is repeated.

2. Protein denaturation and DNA purification

Following deparaffinization, 700 µl of lysis buffer-I (1X) was added to the tissue core taken in microcentrifuge tube and kept in ice for 15 minutes. The tube was removed from ice, shaken vigorously and centrifuged at 10,000 rpm for 15 minutes. A pellet was formed at the bottom of tube and the supernatant was discarded. Again, the addition of 700 µl of of lysis buffer-I (1X) was made and the tube was kept in ice for 15 minutes. After 15 minutes, tube was recovered from ice bath, shaken vigorously and centrifuged at 10,000 rpm for 15 minutes.

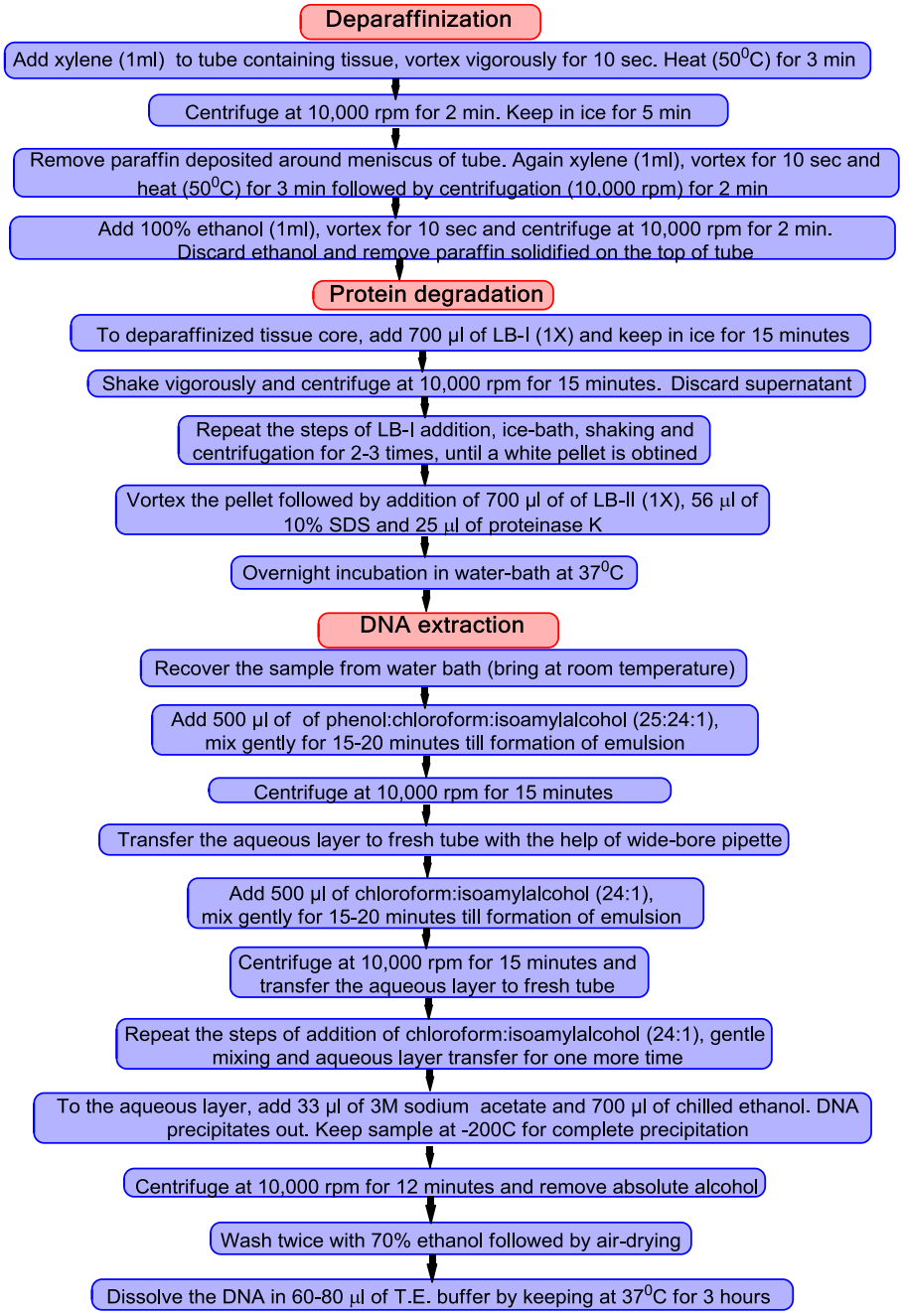


Fig. 15: DNA isolation procedure from FFPE tissue samples

Supernatant was discarded, leaving the pellet behind. The process was repeated for 1-2 times, until a white pellet was obtained. Vortex was done and; 700 µl of of lysis buffer-II (1X), 56 µl of 10% SDS and 25 µl of proteinase K were added. Sample was placed in water bath at 37^{0}C for overnight incubation. Next day, sample was recovered from water bath and allowed to come at room temperature.

500 µl of phenol:chloroform:isoamylalcohol in the ratio of 25:24:1 was added to this followed by gentle mixing for 15-20 minutes till emulsion formation took place. The emulsion was centrifuged at 10,000 rpm for 15 minutes. The aqueous layer was transferred to fresh tube with the help of wide-bore tip. To this, 500 µl of chloroform:isoamylalcohol in the ratio of 24:1 was added, mixed gently for 15-20 minutes at rocking shaker and centrifuged at 10,000 rpm for 15 minutes. With the help of wide-bore tip, aqueous layer was transferred to fresh tube followed by addition of chloroform:isoamylalcohol (24:1) in equal volume, gentle mixing 15-20 minutes and centrifugation at 10,000 rpm for 15 minutes. To the aqueous phase, 33 µl of of 3M sodium acetate and 700 µl of chilled ethanol was added and mixed slowly, till DNA precipitation took place. Sample was kept at -20^{0}C for complete DNA precipitation. Then, DNA was centrifuged at 10,000 rpm for 12 minutes. Absolute alcohol was removed from it and washed twice with 70% ethanol followed by air-drying to remove traces of alcohol. DNA was dissolved in 60-80 µl of T.E. buffer by keeping at 37^{0}C for 3 hours. For prolonged storage, isolated DNA samples were kept at -20^{0}C (Fig. 15).

3.10. Quantitative analysis of DNA using Thermo Scientific NanoDrop™ 1000 spectrophotometer

Principle

Just 1µl of sample is enough for quantification using NanoDrop that too with high accuracy and reproducibility. Sample is pipetted onto the end of fiber optic cable (the receiving fiber). The liquid sample then comes in contact with a second fiber optic cable (the source fiber) causing the liquid to bridge the gap between the fiber optic ends. Light coming from a pulsed xenon flash lamp is allowed to pass through the sample. The spectrophotometer utilizing a linear CCD array analyzed the light after passing through the sample. Computer-based software controls the instrument. The data can be procured from archive file in the computer connected to the instrument.

Procedure

1. Using distilled water, the lower measurement pedestal was cleaned and wiped off with clean lint-free wipes.

2. 1µl of the buffer was used for taking an initial blank measurement was taken with, that gets recorded on the computer.

3. Sampling arm was opened, wiped with lint free wipes and 1µl of sample DNA was pipetted out onto the lower measurement pedestal.

4. After closing the sampling arm, spectral measurement was recorded using software installed specific for Nanodrop.

3.11. Qualitative analysis of DNA using Agarose Gel Electrophoresis

Agarose gel electrophoresis is the most conventional method for analysis of DNA. This technique makes use of agarose-based matrix for the separation and purification of macromolecules like proteins and nucleic acids those differ in terms of size, charge or conformation.

Principle

The mobility of migration of linear DNA fragments through agarose gels is inversely proportional to the $\log_{10}$ of their molecular weight. DNA fragments of different sizes can be resolved using agarose gels of different concentrations. High concentration agarose gels are used for the separation of small DNA fragments, while low concentration gels allow resolution of larger DNAs.

An electric field is applied and the negatively-charged DNA is forced to move through the matrix of agarose. This causes resolution of DNA fragments based upon their size. Large DNA fragments move slower than small DNA fragments. Agarose is stained with Ethidium bromide. Ethidium bromide intercalates in between DNA strands, whose excitation in the presence of UV rays aids in the visualization of DNA fragments.

Reagents:

- 1X TAE Buffer
- Ethidium bromide = 2 µl
- Agarose
- Genomic DNA = 0.8%.
- PCR-RFLP = 2%

Requirements:

- Weighing Balance
- Conical flask
- Microwave

- Gel box with comb
- Gel documentation unit

Procedure:

Step 1: Mixing Gel

1. 0.8 grams of agarose was weighed onto a piece of butter paper and added to the conical flask.

2. 100mL of 1X TAE was added to the agarose in the conical flask.

3. Flask was shaken gently for thorough mixing of agarose.

Step 2: Melting Gel

1. Flask containing mixture of agarose and 1X TAE was heated in microwave until a clear mixture was obtained.

2. 2µl of Ethidium Bromide was added to the flask followed by proper mixing.

Step 3: Pouring the Gel

1. A casting tray was cleaned with ethanol and combs were pre-positioned, before pouring the gel.

2. Poured the agarose gel of 5-7mm thickness into the casting tray.

3. It takes at least 30 min for the gel to get solidify.

4. Combs were removed/pulled out after solidification of the gel, leaving behind depressions/wells meant for sample loading.

5. Gel was placed in the gel electrophoretic tank, with wells towards the left (cathode) side. Enough 1X TAE buffer is poured into the electrophoretic tank such that it covers the entire gel.

Step 4: Loading the Gel

1. Sample (genomic DNA/PCR product/RFLP product) was pipetted out, mixed properly with 2 µl of dye and loaded into the particular well.

Note: 5 µl of 100 bp ladder was also loaded in one of the wells, while analyzing the PCR or RFLP products.

2. Electrophoretic tank was covered. Power supply was turned-on and gel was run for 30 minutes at 80 volts.

Step 5: Visualizing the Gel

The gel was visualized in the presence of UV rays using Bio Rad gel doc.

3.12. DNA dilution

All the DNA samples were diluted with T.E. buffer to achieve a final concentration of 10-15 ng/µl. Working dilutions were run on 0.8% agarose gel.

3.13. Genotyping

3.13.1. Screening of genomic alterations using Global Screening Array

Genotyping was performed on Illumina Infinium HD assay platform using Global Screening Array (GSA) microchip (Illumina Inc.) with 200 ng of genomic DNA as per manufacturer's instructions. DNA collected from blood samples of 60 patients and 20 controls were subjected to GSA. In addition, 20 tissue samples of the patients who were on trastuzumab therapy were also analyzed by GSA. GSA microchip was chosen for study due to the presence of updated markers (more than 700,000) optimized for human genome-wide backbone for unparalleled genomic coverage, including clinically relevant content and pharm GKB markers. Subsequent sample processing and array hybridization were performed according to the manufacturer's instructions (Illumina, Inc.).

Genotypes were called within Genome-Studio with the Gen Call algorithm of Genotyping Module v1.0. The final sample call rate was 99.99%. The data was subsequently exported to R/Bioconductor to calculate X^2 and odds ratio. Annotation was performed using ClinVar, 1000 Genomes, ExAC, Cosmic and dbSNP databases. The p value $P \leq 5 \times 10^{-8}$ was considered statistically significant.

3.13.2. Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP)

The most common significant germline variant of HER2 (I655V) which emerged from GSA analysis was further evaluated as well as validated by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) in all patient (n=100) and control (n=100) samples including the remaining samples which couldn't be analyzed by GSA on account of financial constraints.

3.13.2.1. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a method used for the gene-specific amplification of DNA. Steps involved in PCR are: denaturation, annealing, extension and final extension. Firstly, the double stranded DNA is denatured and separated into single strands following their annealing with forward and reverse primers. Next step is the extension of the two strands using dNTPs present in the PCR master mix. This

causes the generation of stretches of new strands complementary to the respective template strands. For each annealed primer, a new single strand of DNA is synthesized. Cycles are repeated. Repetition of cycle allows all previously synthesized products to act as templates for new primer extension reactions in each ensuring cycle (Fig. 16).

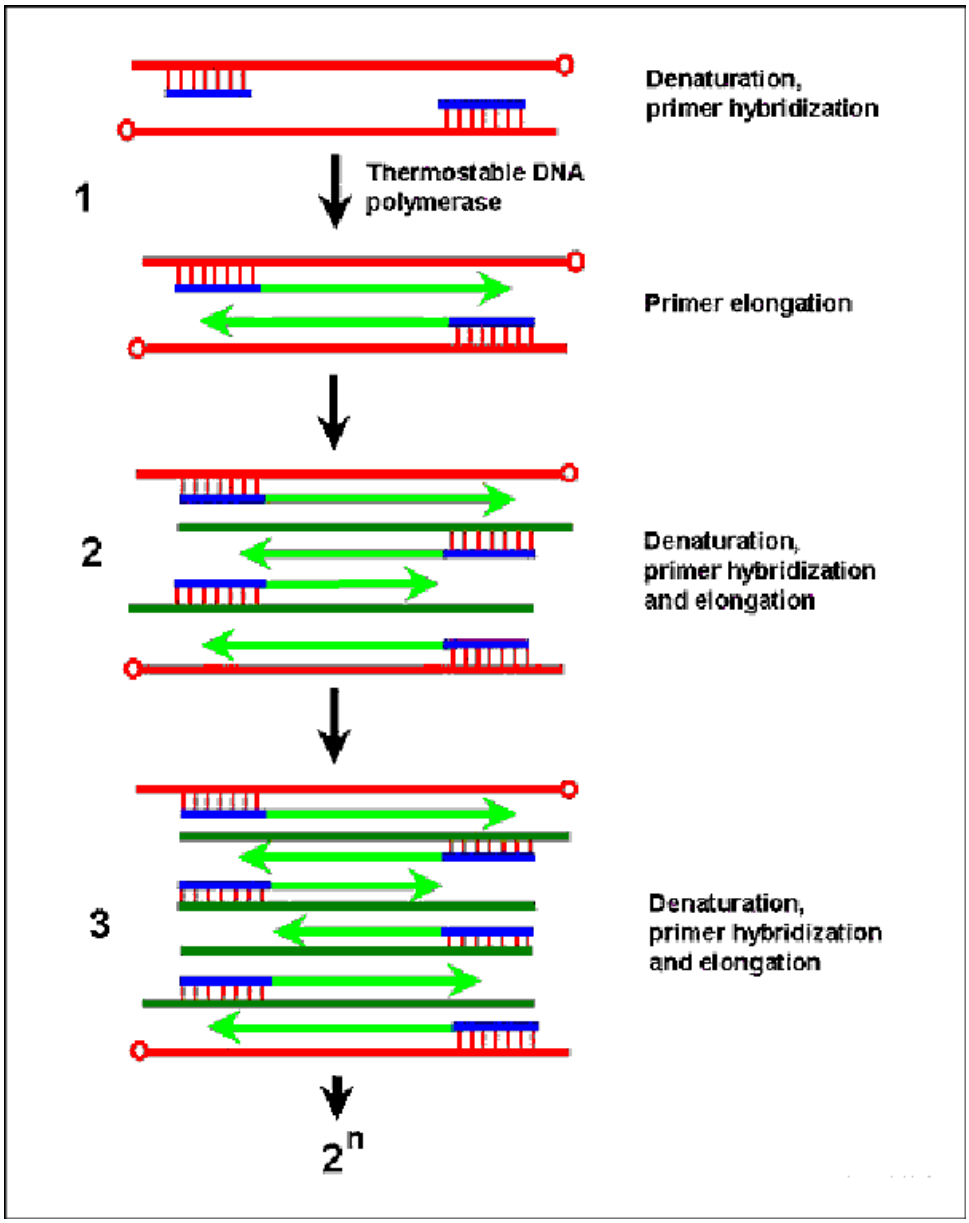


Fig. 16: Schematic representation of PCR steps

Required Equipment

Thermal Cycler, PCR tubes and pipettes

Procedure

1. The contents of PCR master-mix were mixed well using mini-centrifuge. The PCR master-mix composition has been presented in Table 1.
2. Thereafter, PCR tubes having master-mix were placed in thermal cycler for amplification. The PCR conditions have been shown in Table 2.

Table 1: PCR Master-Mix composition for the amplification of HER2 (I655V) polymorphic site

Reagents	**Amount**
Distilled water	35 µl
Taq buffer	5 µl
dNTPs	2 µl
$MgCl_2$	5 µl
Forward Primer (F-5'AGA GCG CCA GCC CTC TGA CGT CCA T3')	0.5µl
Reverse Primer (R-5'TCC GTT TCC TGC AGC AGT CTC CGC A3')	0.5µl
Taq polymerase	1µl
Template DNA	3 µl
Total	50 µl

Table 2: PCR conditions for HER2 (I655V) polymorphic site

	PCR Steps	**Temp.**	**Time**
Step 1	Initial denaturation	94°	30 sec
Step 2	Denaturation	94°	30 sec
Step 3	Annealing	62°	30 sec
Step 4	Extension	72°	30 sec
	Repeat step 2-4 for 35 cycles		
Step 5	Final extension	72°	7 min
	Hold at 4°C for 1 Hour		

3.13.2.2. Restriction Fragment Length Polymorphism (RFLP)

RFLP employs restriction endonucleases those recognize specific sequences and cut double stranded DNA within their recognition sequence to produce fragments. It is a widely used technique for the detection of known mutations and

variations. Fragments produce of as a result of RFLP are resolved by agarose gel electrophoresis and are visualized under the UV rays to analyze the sequence variations of discrete region. BsmA1 restriction enzyme was used for the digestion of 148 bp PCR product.

Required Equipment

- Heat block
- Horizontal slab gel electrophoresis apparatus
- Gel Documentation apparatus
- 0.2ml PCR tubes

Procedure

1. The components were mixed well using mini-centrifuge and placed for overnight incubation at 37^0C in a heat block. RFLP reagent composition has been shown in Table 3.

Table 3: Reagents for RFLP

Reagent	Volume
Nuclease-free water	17µl
10X fast-digest buffer	2µl
PCR product	10µl
Restriction enzyme	1µl
Total Volume	30µl

2. The restriction digested products were separated on 2% Agarose gel.
3. The results were analyzed by visualizing the gel using Bio Rad gel doc.

3.14. Statistical analysis

Association of the disease and the HER2 (I655V) with various demographic/clinico-pathological features was estimated by odds risk ratio with 95% confidence interval (CI) and χ2 analysis using Open Epi software (Open Epi version 2.3.1 from Department of Epidemiology, Rollins School of public health, Emory University, Atlanta, GA 30322, USA). Hardy-Weinberg equilibrium (HWE) was tested for HER2 (I655V) polymorphism.

Kaplan-Meier method and log-rank test performed were using SPSS (Chicago, IL, USA) to determine the association of gene variants with disease-free survival (DFS) of patients in response to trastuzumab treatment. A p-value ≤ 0.05 was DFS considered significant, here.

3.15. Design, synthesis and biological evaluation of anilinoquinazolines

3.15.1. General: Synthesis

All the reagents and solvents were purchased from Sigma-Aldrich, Loba-Chemie Pvt. Ltd., S.D.F.C.L., Sisco Research Laboratory and HiMedia Laboratories Ltd. (AR/GR quality) and were used without further purification. Sartorius analytical balance (BSA224S - CW) was used for the weighing purposes. Biotage® Initiator microwave synthesizer (Company: Biotage® Model No. 355301 (Initiator EXPEU)) (used for sealed reactions) and Discover System; Company: CEM; Model No. 908010; Serial No. DU9671) (used for open reflux reactions) were used for carrying out reactions under microwave irradiations at 200 W power. JSGW heating mantle for reflux reactions and ILMVAC rotary evaporator, were used for evaporating the organic solvents. The progress of the reactions was monitored by TLC, using either petroleum ether/ethyl acetate or chloroform/methanol as the mobile phase on pre-coated Merck TLC plates in JSGW UV/fluorescent analysis cabinet and/or iodine chamber. NMR experiments were recorded at IIT, Ropar Punjab (400 MHz Jeol NMR spectrophotometer). Mass (EI) spectra were recorded on Shimadzu QP 2010 Ultra GCMS at Central University of Punjab.

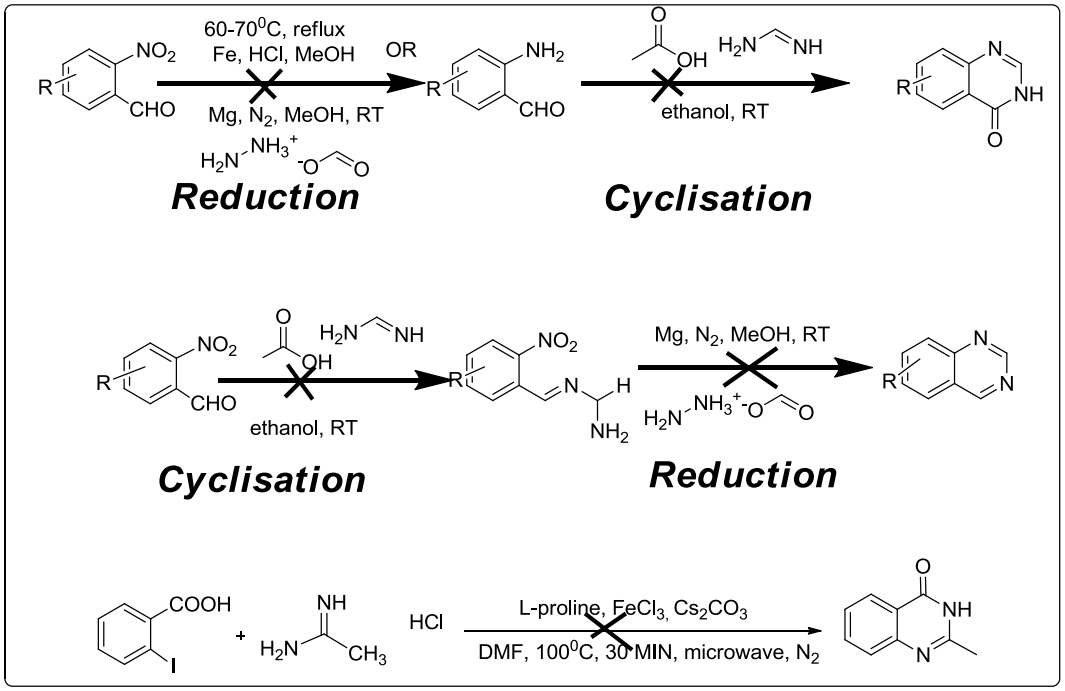


Fig. 17: Routes failed to yield anilinoquinazolines

3.15.1.1. Different routes tried for synthesis of anilinoquinazolines

Reactions involving the reduction of 2-nitrobenzaldehyde followed by cyclization with formamidine acetate to from quinazolone and other way round involving cyclization followed by reduction were tried. However, these reactions failed to yield desired product. Then, a one-pot microwave-assisted reaction involving 2-iodobenzoic acid and acetamidine was tried that also didn't work (Fig. 17).

3.15.1.2. Modification in scheme: Synthesis of pyrimidines

Following the failure of synthesis of anilinoquinazolines, pyrimidine-based derivatives were tried to be synthesized. Different schemes (scheme I, II and III) tried to synthesize pyrimidines have been shown in Fig. 18, 19 and 20. Chlorination reaction didn't succeed; while following scheme I (Fig. 18). In scheme II, a lot of side-products were formed causing inability to purify the desired product (Fig. 19). The same problem of purification also persisted in scheme III, where we failed to obtain pure product even after rigorous column purification (Fig. 20).

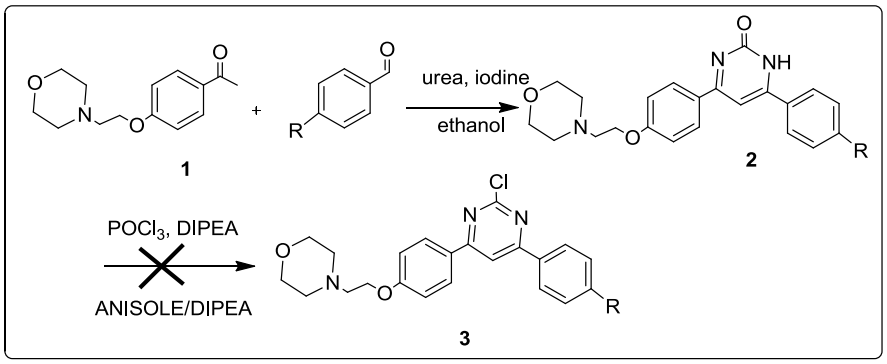


Fig. 18: Scheme I for synthesis of pyrimidine-based compounds

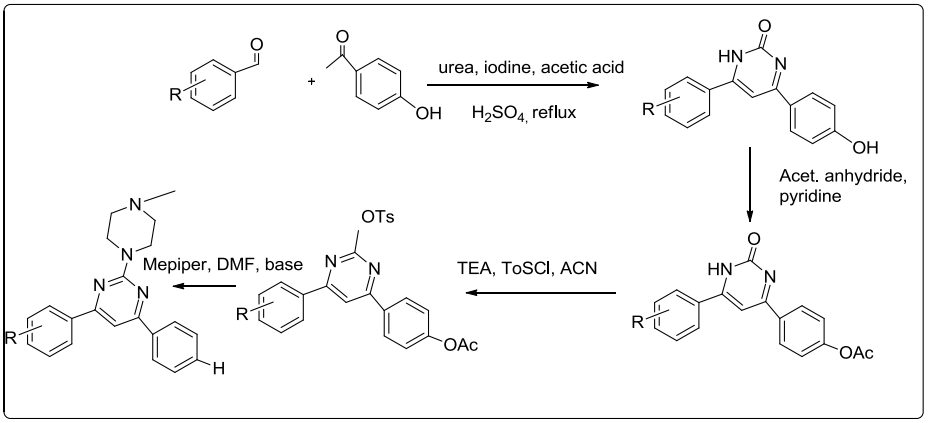


Fig. 19: Scheme II for synthesis of pyrimidine-based compounds

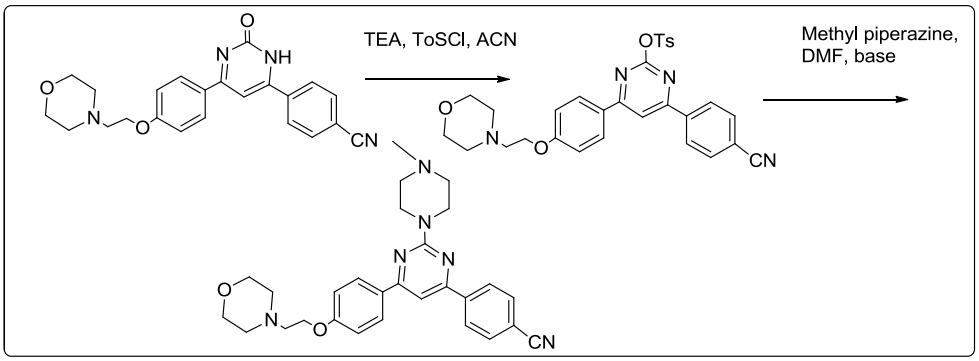


Fig. 20: Scheme III for synthesis of pyrimidine-based compounds

3.15.1.3. Synthesis of quinazoline-based compounds

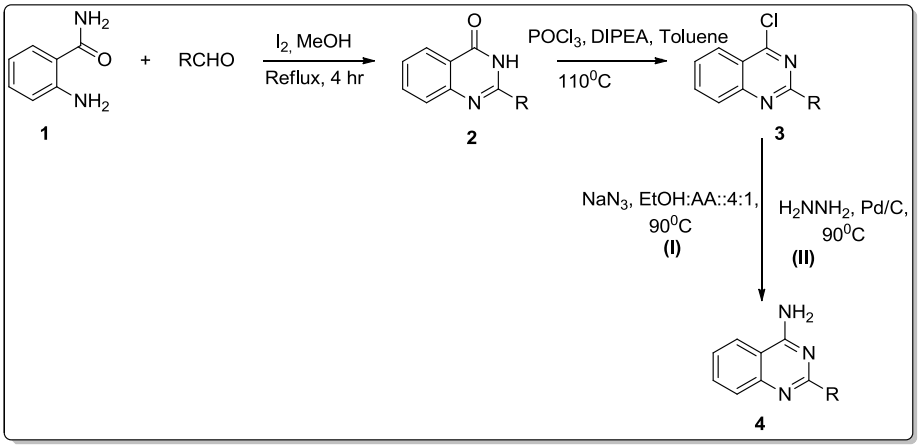


Fig. 21: Synthetic scheme for synthesis of final compounds

Quinazoline-based compounds were synthesized through another route using anthranilamide and benzaldehyde as starting material (Fig. 21), following the failure to get pyrimidine-based derivatives. The detailed synthetic strategy has been discussed ahead.

Synthetic strategy:

Step 1: Synthesis of quinazolinone (2):

To substituted aldehyde (1eq), anthranilamide was added, along with addition of iodine (1eq) as oxidative catalyst and methanol (10ml) as solvent. The reaction mixture was stirred for 4h at 80^0C. The completion of the reaction was monitored via TLC. After completion of reaction, the excess of solvent was evaporated from mixture and concentrated under vacuum using rotary evaporator. The chilled water was poured in reaction mixture and the precipitates obtained were filtered and dried.

Step 2 : Chlorination of quinazolinone (3):

To quinazolinone (2) (1g), phosphoryl trichloride (1.2eq) was added along with the addition of 2 drops of N,N-Diisopropylethylamine (DIPEA) as base and toluene as solvent. The reaction mixture was refluxed at 110^{o}C, till completion. The reaction process was monitored *via* TLC. After completion of reaction the excess amount of ice was kept into the reaction mixture followed by neutralization with sodium bicarbonate. The reaction mixture was extracted with chloroform (20ml x3), and washed with water and brine solution. The organic layer was dried over anhydrous sodium sulphate and then, concentrated under vacuum using rotary evaporator.

Step 3 : Synthesis of final compound (quinazoline-4-amine, (4))

To synthesize amino-quinazoline (4), chlorinated quinazoline (3) was heated with sodium azide (NaN_3) in ethanol:glacial acetic acid::3:1 for 2 h at 90^{0}C. The contents were then cooled down followed by the addition of 10 mol.% of Pd/C (10%) and subsequent slow addition of 1.5 eq. of hydrazine ($H_2NNH_2.H_2O$). The reaction mixture was re-heated at 90^{0}C for 2 h. Monitoring of the reaction completion was done *via* TLC. After reaction completion, Pd/C catalyst was filtered off the reaction mixture, followed by removal of the ethanol/acetic acid in vacuum followed by purification using column chromatography.

3.15.2. Biological evaluation

3.15.2.1. Cell culture and treatment

All the cell lines including HER2-positive breast cancer cell line (SKBR3), triple-negative breast cancer cell line (MDA-MB-231) and normal breast epithelial cells (FR-2) were procured from National cell repository situated at NCCS, Pune. Cell lines were grown in DMEM media supplemented with 10% fetal bovine serum (FBS) and antibiotic solution (1X Penstrip, all the reagents from Invitrogen). The cells were incubated at 37 ^{o}C with 5 % CO_2 and 95 % humidity conditions. For experiments, cells were seeded in equal numbers after trypan blue cell counting (5,000 cells per well of 96-well plate and 100,000 cells per well of 6 well plate). Afterwards cells were washed once with sterile 1 X PBS and cultured with serum free media for 8 h for synchronization. The test compounds were dissolved in cell culture grade DMSO upto concentration of 100 mM and further dilutions were done in serum free DMEM media. The total amount of media per well (200 µL

per well of 96 well plate and 2 mL per well for six well plates) was kept constant and all the treatment volumes were accommodated within these ranges only.

The cancer cell lines were cultured in DMEM medium supplemented with 10% FBS and antibiotic solution followed by trypsinization for sub-culturing. Subsequently, the inactivation of the trypsin enzyme was brought about by harvesting the cells in 5 ml media containing serum, followed by the centrifugation of the harvested cells at 1200 rpm at 4^0C for 5 minutes. The obtained supernatant was discarded and the pellet was re-suspended in 2 ml of media. The automated cell counter was used to count the cell number. After every three days, the cells were transferred to fresh media.

3.15.2.2. 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

MTT assay was carried out using 96-wellplate; each well was filled by 100 µL media to which cell were treated and subsequently washed with 1 % PBS and were mixed with 100 µL/mL well of MTT (5 mg in 10 mL of 1 % PBS) and incubated at room temperature in dark for 4 h to allow formation of formazan crystals. Each well was then mixed with 100 µL of DMSO to dissolve the crystals followed by readings using microplate reader at 570 nm. The results were then represented as mean ± S.D obtained from three independent experiments.

CHAPTER 4

RESULTS

4.0. FISH interpretation

As per FISH evaluation, 10 out of 19 equivocal cases were found to be positive for HER2/neu gene amplification, therefore accounting for a total no. of 100 HER2-positive breast cancer cases (90 IHC3+ and 10 FISH+) (Fig. 22).

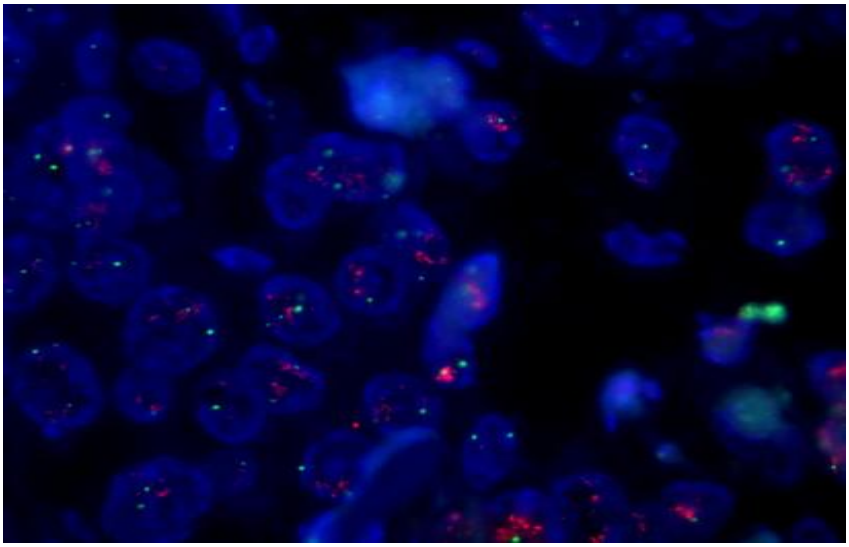


Fig. 22: FISH analysis of a HER2-positive breast cancer specimen. Orange signals for HER2/neu gene locus (17q11.2-q12) and green signals for CEP17

4.1. Demographic Profile

In this case-control study, 100 clinically confirmed female HER2-positive breast cancer patients from Malwa region of Punjab and 100 age and ethnicity-matched healthy female controls were included. The data pertaining to demography was recorded in specially-designed performa.

4.1.1. Age

Mean age of the patients at the time of diagnosis was 53.23±12.1 and at the time of menarche and first pregnancy, it was observed to be 14.92±1.32 and 22.05±2.42, respectively. 22% (22/100) of the patients had their first pregnancy after 25 years. Earlier menarche (<14 years) was observed in 35% (35/100) of the patients.

Mean age of the control group was 53.14±11.9. At the time of menarche and first pregnancy, the mean age was recorded to be 13.81±1.21 and 21.92±2.12, respectively. Earlier menarche (<14 years) and late first pregnancy

(>25 years) were recorded in 42% (42/100) and 39% (39/100) of the controls, respectively.

4.1.2. Locality

52% (52/100) of patients were from the rural background and 48% (48/100) were residing in the urban area**.** In the study control group, 42% (42/100) lived in urban area and the rest 58% (58/100) resided in rural locality (Fig. 23).

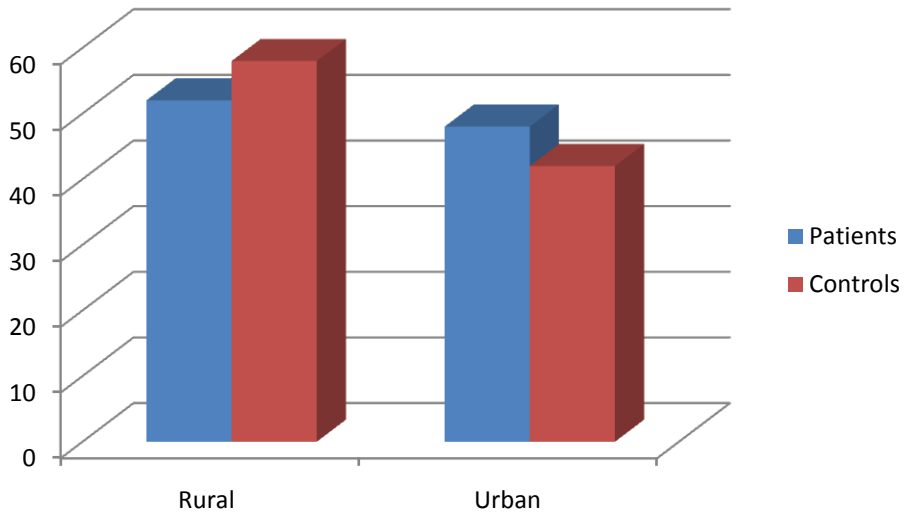


Fig. 23: Number of patients and controls of rural and urban locality

4.1.3. Menopausal Status

Postmenopausal HER2-positive breast cancer was observed in 46% (46/100) of the females, whereas 39% (39/100) of the patients were premenopausal at the time of diagnosis. 15% (15/100) of the patients had undergone hysterectomy, earlier (Fig. 24).

As far as the controls are concerned, 45% (45/100) were premenopausal and 51% (51/100) were postmenopausal. Remaining 4% (4/100) had undergone hysterectomy (Fig. 24).

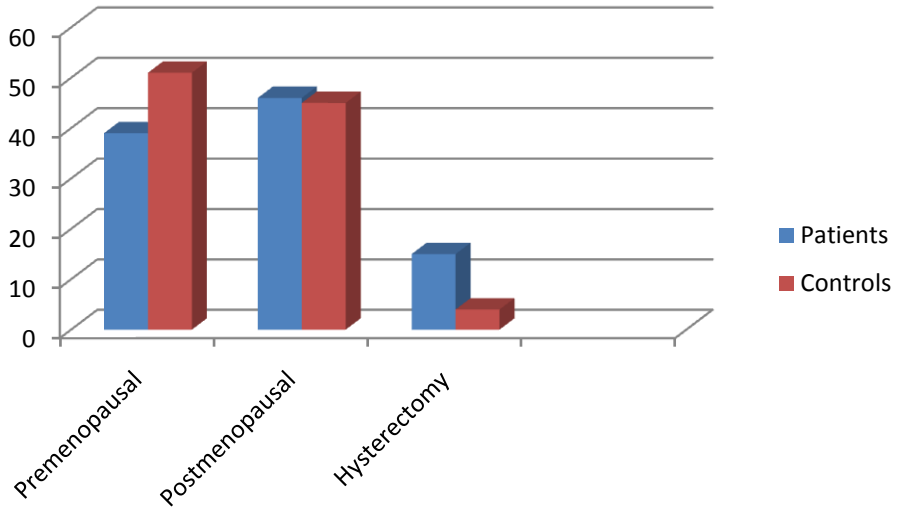


Fig. 24: Menopausal status of patients and controls

4.1.4. Obesity

53% (53/100) of the patients were obese, whereas 42% (42/100) displayed normal BMI. The remaining 5% (5/100) were underweight. In case of controls, 39% (39/100) had normal BMI, whereas 61% (61/100) were obese (Fig. 25).

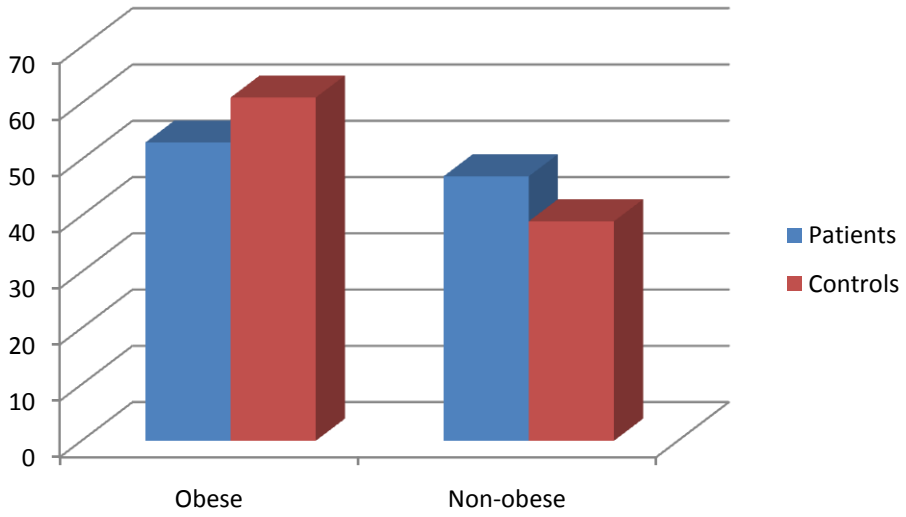


Fig. 25: Obesity among patients and controls

4.1.5. Family history of breast and other cancers

The family history of cancer including breast and other cancers was observed in 8% (8/100) of the patients. None of the controls had personal/familial history of breast and other cancers.

4.1.6. Pesticide exposure

Since Punjab is a leading grain producer in India with maximum use of pesticides, around 43% (43/100) patients had an exposure to pesticides, whereas 47% (47/100) of the controls had pesticide-exposure (Fig. 26).

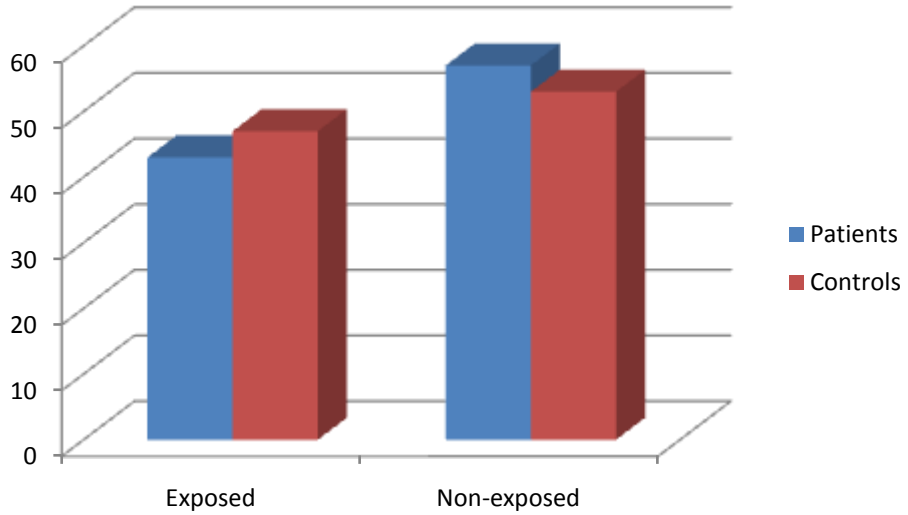


Fig. 26: Pesticide exposure among patients and controls

Demographic profile of patients and controls has been given in Table 4. None of the demographic features had significant association with HER2-positive breast cancer.

Table 4: Comparison of demographic characteristics between HER2-positive breast cancer patients and healthy controls

Characteristics	Patients	Controls	p
Age at first pregnancy			
≥ 25 years/	22	39	>0.05
<25 years	78	61	
Age at menarche			
< 14 years/	35	42	>0.05
≥ 14 years	65	58	
Menopausal status			
Premenopausal/	39	45	>0.05
Postmenopausal +	46	51	
Hysterectomy cases	15	4	

Locality			
Urban/	48	42	>0.05
Rural	52	58	
Obesity			
Yes/	53	61	>0.05
No	47	39	
Pesticide Exposure			
Yes/	43	47	>0.05
No	57	53	

4.2. Follow-up

Out of 100 HER2-positive breast cancer patients, 70 received trastuzumab treatment. Death, recurrence and metastasis were observed in 15.71% (11/100), 11.43% (8/70) and 22.86% (16/70), respectively. However, in 20 patients whose tissue samples were also analyzed 15% (3/20), 10% (2/20) and 20% (4/20) of the patients suffered from death, recurrence and metastasis, respectively. On the basis of death, recurrence and metastasis, these patients were classified into two groups: patients with good clinical outcome and patients with bad clinical outcome.

4.3. Molecular analysis

DNA was isolated from the blood and FFPE tissue samples collected from the subjects. DNA isolated was analysed quantitatively and qualitatively. The concentration of DNA isolated from blood and tissue was 250-1500 ng/µl and 100-320 ng/µl respectively. The qualitative analysis was carried out by gel electrophoresis and the intact bands revealed the good quality of DNA (Figs. 27 and 28). All the DNA samples were diluted to achieve a final concentration of 10 ng/µl.

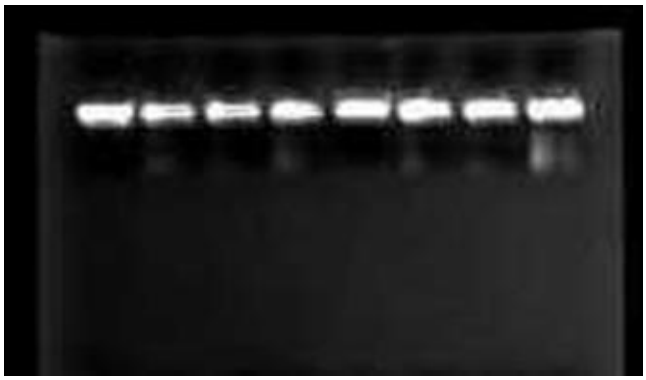


Fig. 27: Agarose gel electrophoresis of genomic DNA isolated from blood

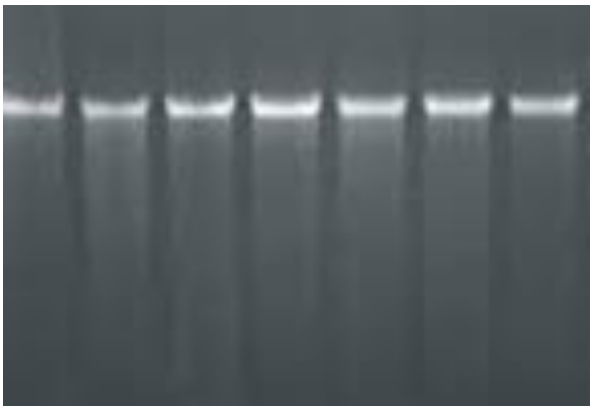


Fig. 28: Agarose gel electrophoresis showing genomic DNA isolated from tissue

4.4. Evaluation of genomic alterations

4.4.1. Germline variants

In HER2-positive breast cancer patients (n=60) and age-matched healthy female controls (n=20), HER2 and other genes namely HER1, ESR1, KRAS, VEGFA, TP53, PIK3CA, IL6, CYP2C19, CYPA452, CYP3A5, CYP2A7P1, CYP2B6, CYP3A4, CYP2C9, CYP2C8, CYP4A2, CYP19A1, CYP4B1, CYP2D6, CYP7A1, CYP2E1, CYP3A5,CYP2A6, CYP1A2 and CYP11B2 were screened by GSA.

A germline polymorphism in HER2 (I655V) was found to be significantly associated with HER2-positive breast cancer [($p<0.01$, GG vs AA : Odds Ratio: 7.008 (2.742-17.91); GG+AG vs GG: Odds ratio: 4.462 (2.2-9.046)], also confirmed by PCR-RFLP in all patients (n=100) and controls (n=100) (Fig. 29). The distribution of HER2 I655V polymorphism in HER2-positive breast cancer patients and controls has been presented in Table 5. These

results indicate Val allele to be significantly associated with HER2-positive breast cancer.

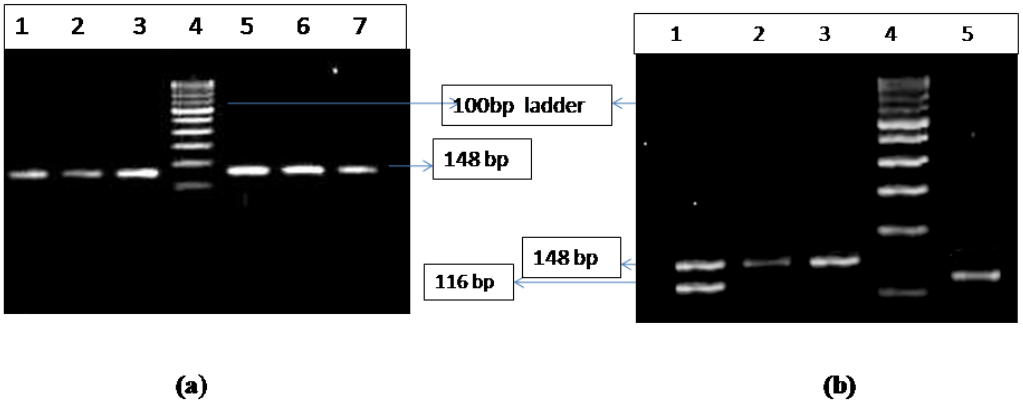


Fig. 29: (a) Gel picture showing PCR amplification. Lanes 1-3 and 5-7 show amplified product and lane 4 represents 100bp ladder. (b) Restriction fragment length polymorphism of the HER2 I655V gene polymorphism. Lane 1 represents heterozygous condition; Lanes 2 and 3 represent homozygous wild type and lane 5 homozygous mutant genotype

Further, HER2 (I655V) was found to be significantly associated with pesticide exposure ($p<0.05$; Table 6). However, no significant association was found between HER2 (I655V) genotype and other demographic features of HER2-positive breast cancer (Table 6).

Table 5: Association of HER2 (I655V) polymorphism with HER2-positive breast cancer

	AA(Ile/Ile)	**AG(Ile/Val)**	**GG(Val/Val)**	**p(GG vs AA)**	**p(GG+AG vs AA)**	**A (Ile)**	**G (Val)**	**p(G vs A)**
Patients	60	11	29	<0.01	<0.01	131	69	<0.01
Controls	87	7	6			181	19	

Table 6: Association of HER2 I655V polymorphism with demographic features of HER2-positive breast cancer

Characteristics	**AA (Ile/Ile)**	**AG+GG (Ile/Val +Val/Val)**	**p**
Age at diagnosis			
≤45 years (n=45)/	23 (51.11%)	22 (48.89%)	>0.05
>45 years (n=55)	37 (67.27%)	18 (37.73%)	
Age at first pregnancy			
≥25 years (n=22)/	12 (54.55%)	10 (45.55%)	>0.05
<25 years (n=78)	48 (61.54%)	30 (38.46%)	
Age at menarche			
≥14 years (n=35)/	24 (68.57%)	11 (31.43%)	>0.05
<14 years (n=65)	36 (55.38%)	29 (44.62%)	
Menopausal status			
Premenopausal (n=39)/	25 (64.1%)	14 (35.9%)	>0.05
Postmenopausal (n=46)	35 (57.38%)	26 (42.62%)	
+Hysterectomy cases(n=15)			
BC Family History			
Yes (n=5)/	3 (60%)	2 (40%)	>0.05
No (n=95)	57 (60%)	38 (40%)	
Obesity			
Yes (n=53)/	32 (60.38%)	21 (39.62%)	>0.05
No (n=47)	28 (59.57%)	19 (40.43%)	
Pesticide exposure			
Yes (n=43)/	18 (41.86%)	25 (58.14%)	<0.01
No (n=57)	42 (73.68%)	15 (26.32%)	

4.4.2. Evaluation of somatic mutations

A PTPN1 nonsense mutation (K99X), a pathogenic CCND1 splice site variant (P241P), a hotspot missense mutation in PIK3CA (E542K) and a hotspot missense mutation in TP53 (R249S) were observed in 25%, 75%, 30% and 40% of patients respectively.

4.4.3. Association with clinical outcome in response to Trastuzumab

CCND1 (P241P) and PIK3CA (E542K) were found to be significantly associated with poor clinical outcome in HER2-positive breast cancer patients, who were on trastuzumab-therapy (p=0.018 and 0.005, respectively). A significantly lower DFS was observed in HER2-positive breast cancer patients treated with trastuzumab, who harbored mutant CCND1 and/or PIK3CA than those carrying wild-type CCND1 and/or PIK3CA (Figs. 30 and 31).

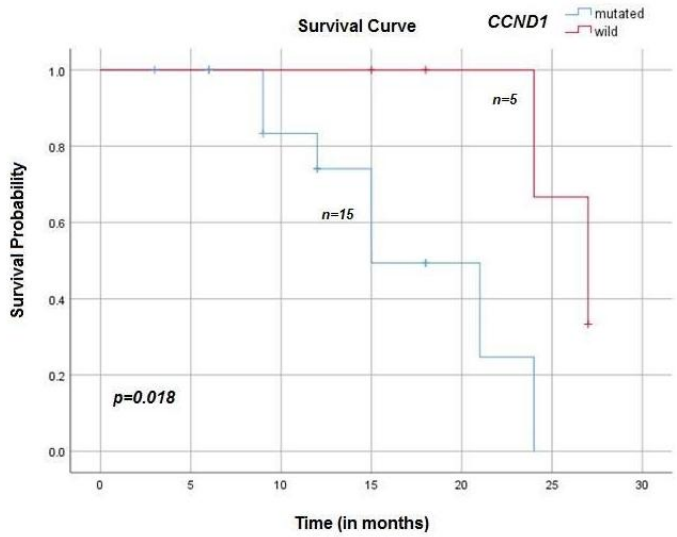


Fig. 30: Kaplan-Meier plot showing DFS according to CCND1 (P241P) mutation status in HER2-positive breast cancer patients treated with trastuzumab

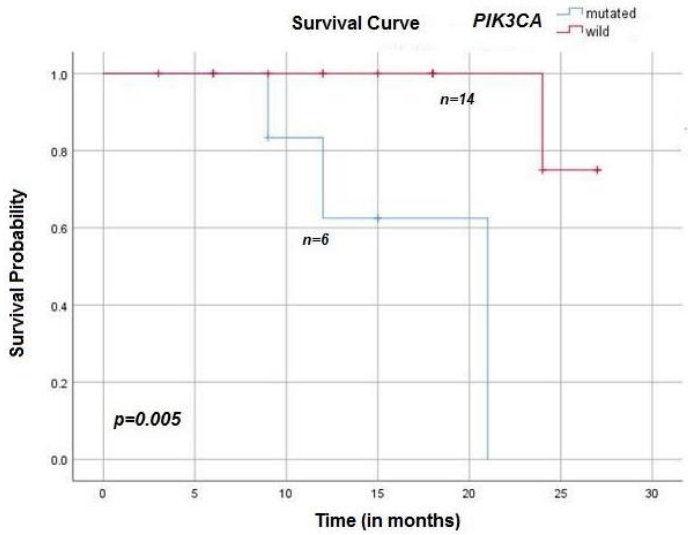


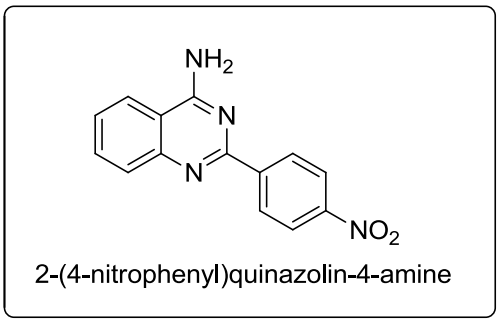
Fig. 31: Kaplan-Meier plot showing DFS according to PIK3CA (E542K) mutation status in HER2-positive breast cancer patients treated with trastuzumab

Somatic mutations PTPN11 (K99X) and TP53 (R249S); and the germline variant HER2 (I655V) were not found to be significantly associated with clinical outcome in trastuzumab-treated HER2-positive breast cancer (p>0.05).

4.5. Spectral data of synthesized compounds

Five synthetic analogues were synthesized, purified and characterized by mass and NMR data.

4.5.1. 2-(4-nitrophenyl)quinazolin-4-amine

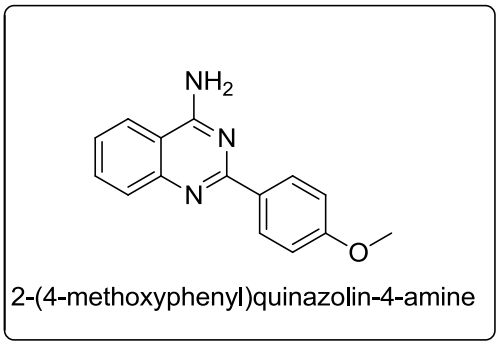


HS-2, m/z=266, Pale-yellow solid, Yield=66%

^{1}H NMR (400 MHz, CHLOROFORM-D, TMS=0) δ 8.07 (d, *J* = 8.7 Hz, 1H), 7.65 (d, *J* = 8.7 Hz, 2H), 7.59 (d, *J* = 8.2 Hz, 1H), 7.25 (s, 1H), 7.18 (d, *J* = 8.2 Hz, 1H), 7.12 (t, *J* = 7.5 Hz, 1H), 6.84 (t, *J* = 7.5 Hz, 1H).

^{13}C NMR (100 MHz, CHLOROFORM-D, TMS=0) δ =162.10, 157.88, 150.07, 148.12, 144.75, 132.70, 128.67, 127.73, 123.37, 122.86, 113.36, 39.78, 39.57, 39.36

4.5.2. 2-(4-methoxyphenyl)quinazoline-4-amine

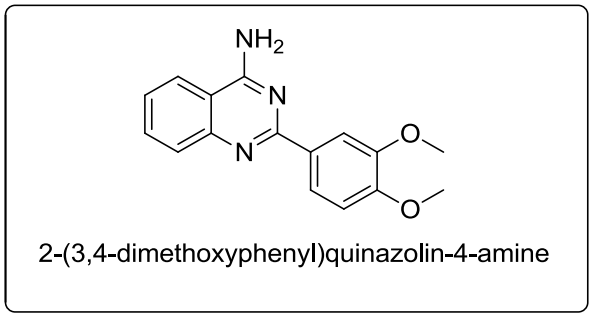


HS-3, m/z=251, Yellow-brown solid, Yield=65%

^{1}H NMR (400 MHz, CHLOROFORM-D, TMS = 0) δ 8.46 (2H, d, *J* = 9.0 Hz), 7.94 – 7.87 (1H, m), 7.76 – 7.67 (2H, m), 7.39-7.43(1H, m), 7.01 (2H, d, *J* = 9.0 Hz), 5.75(2H, s), 3.87 (3H, s).

^{13}C NMR (100 MHz, CHLOROFORM-D, TMS = 0) δ =161.53, 161.39, 160.66, 151.18, 133.29, 130.01, 128.72, 125.42, 121.67, 113.77, 55.45, 29.79.

4.5.3. 2-(3,4-dimethoxyphenyl)quinazoline-4-amine

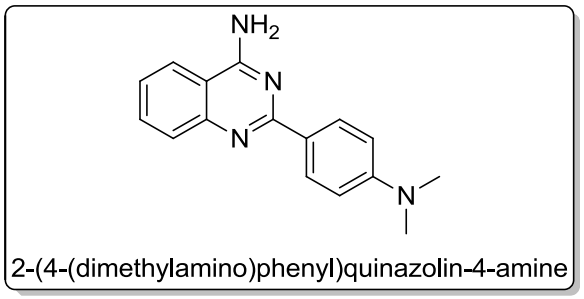


HS-5, m/z=281, Yellow solid, Yield=62%

^{1}H NMR (400 MHz, CHLOROFORM-D, TMS = 0) δ 8.12-8.07(1H, s), 7.92(1H, d, *J* = 12 Hz), 7.79-7.68 (2H, m), 7.40-7.45(1H, m), 6.94 (1H, d, *J* = 12 Hz), 5.70(2H, s), 3.94 (3H, s), 4.02 (3H, s).

^{13}C NMR (100 MHz, CHLOROFORM-D, TMS = 0) δ =161.35, 160.56, 151.16, 151.02, 148.82, 133.32, 128.75, 125.51, 121.67, 111.03, 110.68, 56.03, 29.79.

4.5.4. 2-(4-(dimethylamine)phenyl)quinazoline-4-amine

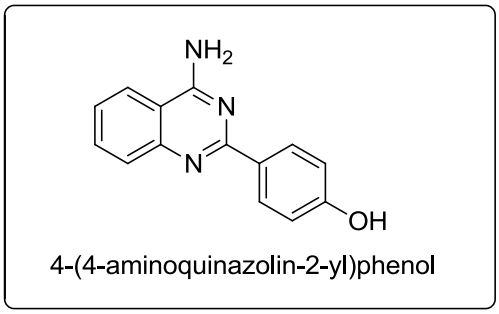


HS-8, m/z=264, Yellow-solid, Yield=69%

^{1}H NMR (400 MHz, CHLOROFORM-D, TMS=0) δ 8.27 (2H, d, J=8Hz), 8.01 (2H, d, J=8Hz), 7.74 (2H, d, J=4Hz), 7.42-7.38(1H, m), 6.79 (2H, d, J=12Hz), 5.29 (2H, s), 3.07 (6H, s).

^{13}C NMR (100 MHz, CHLOROFORM-D, TMS = 0) δ 163.23, 159.00, 152.64, 150.08, 134.80, 128.25, 127.57, 126.46, 125.86, 120.47, 111.81, 40.23.

4.5.5. 4-(4-aminoquinazolin-2-yl)phenol



HS-9, m/z=237, Yellow solid, Yield=64%

^{1}H NMR (400 MHz, DMSO-D6, TMS=0) δ 12.42 (s, 1H), 9.75 (s, 1H), 8.11 (dd, J = 8Hz, 1H), 7.79 (dd, J = 8Hz, 1H), 7.68 (dd, J =Hz, 1H), 7.59 – 7.52 (m, 2H), 7.50 – 7.43 (m, 1H), 7.29 (t, J = 8Hz, 1H), 6.94 (d, J = 8.0Hz, 1H).

^{13}C NMR (100 MHz, DMSO-D6, TMS=0) δ =158.03, 135.14, 130.21, 127.99, 127.07, 126.38, 119.03, 118.86, 115.07.

4.6. Anti-proliferative activity of synthesized analogues

Of the five synthesized compounds (HS-2, HS-3, HS-5, HS-8 and HS-9), three were tested for anti-proliferative activity against HER2-positive (SKBR3), triple-negative (MDA-MB-231) breast cancer cells and normal breast epithelial (FR-2) cells respectively.

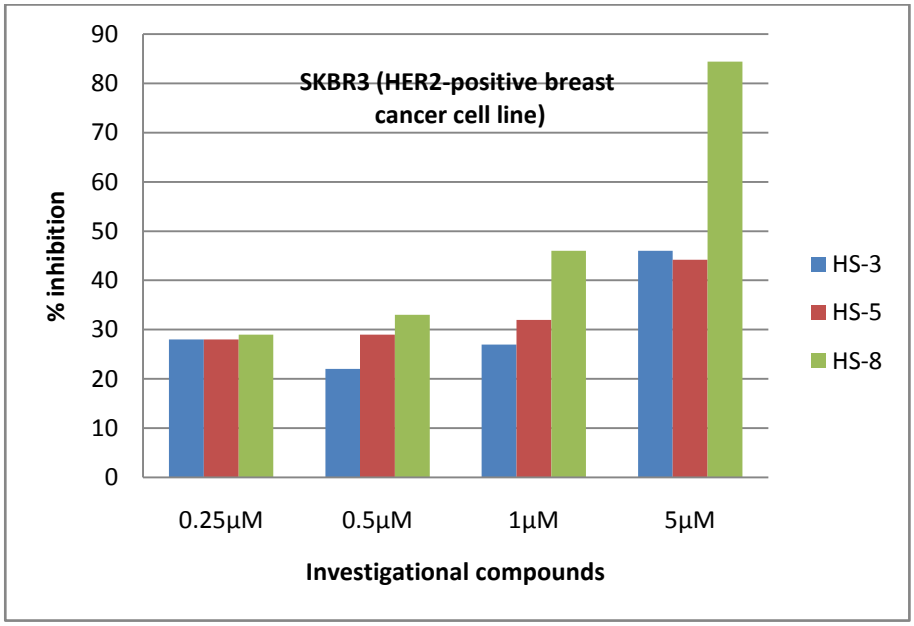


Fig. 32: Percent inhibition of SKBR3 in response to treatment with synthesized compounds at concentrations 0.5, 1 and 5 µM for time duration of 48 hours. Data is expressed as mean values ± S.D.

4.6.1. Anti-proliferative activity against SKBR3 cells

Among the synthesized compounds, HS-8 showed maximum inhibitory effects against SKBR3 cells with an inhibition of 50% between 1-5µM and more than 80% inhibition at 5µM (IC_{50}=2.8µM). Compounds HS-3 and HS-5 inhibited less than 50% of SKBR3 cells even at a higher concentration of 5µM (Fig. 32).

4.6.2. Anti-proliferative activity against MDA-MB-231 cells

HS-8 showed maximum inhibition against MDA-MB-231 cells with an inhibition of more than 40% between 1µM and more than 70% inhibition at 5µM (IC_{50}=3.2µM). On the other hand, HS-3 and HS-5 inhibited less than 45% of SKBR3 cells even at a higher concentration of 5µM (Fig. 33).

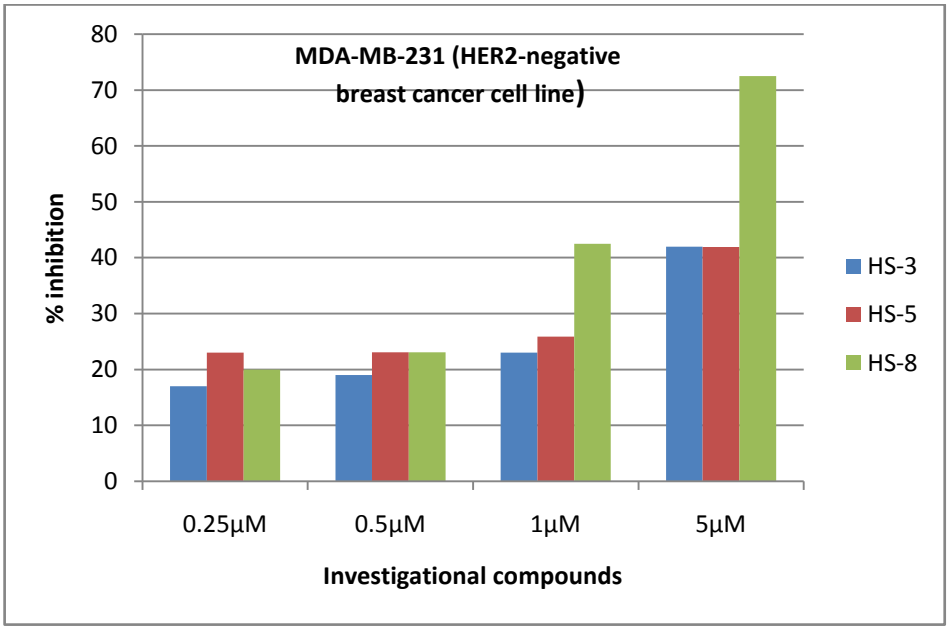


Fig. 33: Percent inhibition of MDA-MB-231 in response to treatment with synthesized compounds at concentrations 0.25, 0.5, 1 and 5 µM for time duration of 48 hours. Data is expressed as mean values ± S.D.

4.6.3. Anti-proliferative activity against FR-2 cells

Compound HS-8 didn't show any cytotoxicity against FR-2 cells, whereas HS-3 and HS-5 exhibited cytotoxicity of >5% and 14%, respectively at a concentration of 5µM (Fig. 34).

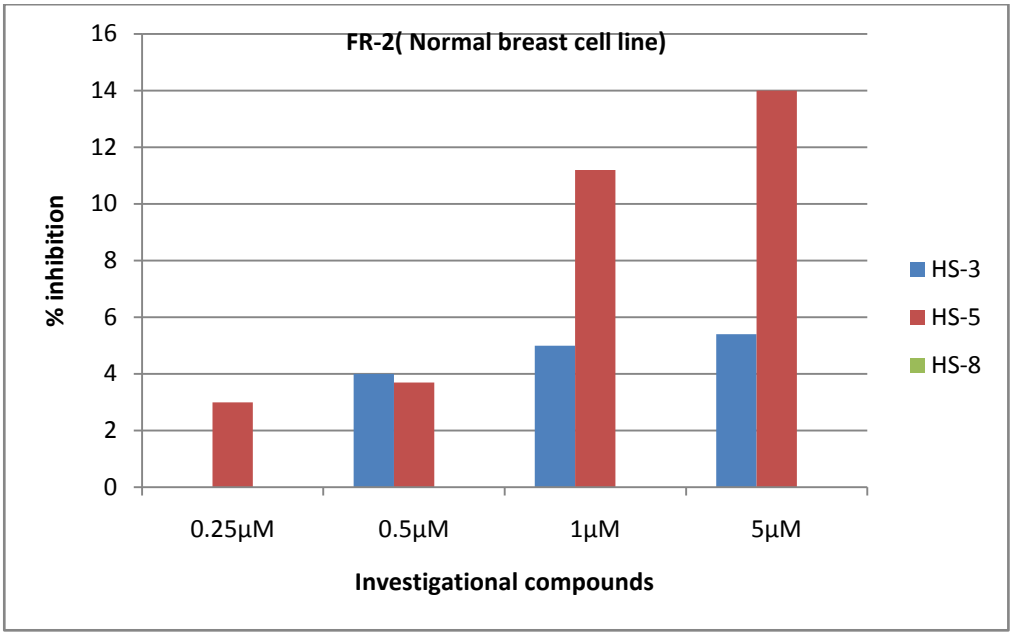


Fig. 34: Percent inhibition of FR-2 in response to treatment with synthesized compounds at concentrations 0.5, 1 and 5 µM for time duration of 48 hours. Data is expressed as mean values ± S.D.

CHAPTER 5
DISCUSSION

5.1. Demographic features

100 female patients confirmed to be affected with HER2-positive breast cancer and 100 healthy age-matched controls from Malwa region of Punjab were included in the study. The current study focused on the evaluation of demographic profile of HER2-positive breast cancer and identification of genomic variants associated with disease-risk and trastuzumab-response. Breast cancer is widely feared in Malwa region and around 20% of the breast cancer cases are found to be affected with HER2-positive subtype. The demographic data including age at diagnosis, age at menarche, age at first pregnancy, rural/urban background, menopausal status, obesity, family history of cancer and pesticide exposure was collected from all the subjects. No significant association of any of these demographic features with HER2-positive breast cancer was observed. In agreement with our study, Li *et al.* also found no association of age at menarche and age at first pregnancy with HER2-positive breast cancer (Li *et al.*, 2013). On the contrary, some studies have reported a significant association of these factors with the disease risk (Phipps *et al.*, 2008; Gaudet *et al.*, 2011; Phipps *et al.*, 2011). This might be on account of differences in ethnicity. However, a systematic review of 38 studies conducted by Barnard *et al.* failed to arrive at any conclusion regarding the risk profile of HER2-positive breast cancer on account of insufficient evidence. They suggested that a greater number of HER2-positive breast cancer cases are required in order to clarify the relationship between established risk factors and HER2-positive breast cancer (Barnard *et al.*, 2015).

5.2. Genomic alterations

Germline alterations were evaluated among 100 HER2-positive breast cancer patients and 100 controls using GSA and PCR-RFLP techniques. Additionally, 20 FFPE tissue samples of HER2-positive breast cancer patients who were on trastuzumab therapy were also screened by GSA. GSA contains approximately 700,000 biomarkers including breast cancer susceptibility genes and drug metabolizing genes. Using GSA, various alterations of HER2 gene and other genes reported previously to be associated with HER2-positive breast cancer or involved in HER2-signaling were evaluated. This is the first study from India, as it

specifically aimed to evaluate genomic alterations implicated in the pathogenesis of HER2-positive breast cancer as well as in conferring response towards trastuzumab-therapy.

5.2.1. Germline variants

HER2 (I655V) is the common HER2 variant that has been detected in blood as well as tumor tissues of BC (Xie *et al.*, 2000; Ferrari *et al.*, 2016; Furrer *et al.*, 2016; Singla *et al.*, 2017). I655V, the germline variant of HER2 gene alters the conformation thereby rendering the receptor continuously active, promoting HER2 homo-dimerisation and tyrosine kinase signaling (Fleishman *et al.*, 2002). The role of Val allele in enhanced cell proliferation is also supported by in vitro experiments that demonstrated that HER2/Val-expressing cells possess a higher growth capacity and lower apoptosis as compared to HER2/Ile-expressing cells, and tumor formation was only seen among HER2/Val-expressing nude mice (Beauclair *et al.*, 2007). Many studies have established a significant association of HER2 (I655V) with breast cancer susceptibility (AbdRaboh *et al.*, 2013; Hou *et al.*, 2013; Singla *et al.*, 2017), whereas others could not establish an association between the two (Sezgin *et al.*, 2011; Nassef *et al.*, 2014). Han *et al.* found that HER2 (I655V) is an important prognostic marker in HER2-positive breast cancer that contributes to more aggressive phenotype. However, Han and colleagues didn't report a significant association of this variant with the disease (X. Han *et al.*, 2014). We identified a significant association of I655V variant with HER2-positive breast cancer. As per the data available based on the personal interviews with patients 43% had pesticide exposure and this associated significantly with HER2 I655V variant. Although we didn't evaluate the presence of pesticides in blood samples, a study conducted by Kaur *et al.* found pesticide residues in the exposed population from Bathinda district (Malwa), as detected by gas chromatography, therefore, indicating that the individuals exposed do harbor some levels of pesticides in their blood (Kaur *et al.*, 2018). Cancer is a multifactorial disease, where both genetic aberrations and environmental factors act in a synergistic manner to promote the disease (Brennan, 2002; Rudolph *et al.*, 2016). The results of the current study suggest that patients carrying HER2 I655V and exposed to pesticides are at a higher risk of developing the disease.

5.2.2. Somatic mutations

As far as the somatic mutations are concerned, PTPN11, CCND1, PIK3CA and TP53 genes were found to be mutated in the study group. PTPN11 has been known to dephosphorylate HER2, hence repressing HER2 signal transduction (Nunes-Xavier *et al.*, 2013; Dittrich *et al.*, 2014). PTPN11 nonsense mutation (K99X) resulting in non-functional protein product, was found in 25% of patients. This non-functional protein may hamper HER2 repressing activities as a result of inhibition of HER2 dephosphorylation, thus, aggravating the effects of HER2-positive breast cancer.

PI3K is a major signaling event, triggered upon HER2 dimerisation. Activated Akt in PI3K signaling cascade leads to increased cell proliferation by transcriptional factor regulation and enhancement of CCND1 levels (Dittrich *et al.*, 2014). In the current study, we found CCND1 (P241P) alteration in 75% of patients. CCND1 (P241P) has been reported to confer an enhanced risk for BC, previously (Lu *et al.*, 2009; Yang *et al.*, 2011). P241P is a synonymous variant at amino acid position 241, in exon 4 splicing site that causes alternate splicing of CCND1 mRNA into transcript-a and transcript-b. The wild-type allele mainly encodes transcript-a, whereas variant allele codes for transcript-b. As transcript-b encoded by the variant has a prolonged half-life, it may lead to enhanced CCND1 protein levels (Betticher *et al.*, 1995). Overexpression of CCND1 permits early G1/S transition in cell-cycle causing abnormal cell proliferation (Pirkmaier *et al.*, 2003).

PIK3CA encodes for p110α, the catalytic component of the PI3K pathway. PIK3CA is found to be frequently mutated in breast cancers (Mukohara, 2015). The current study identified PIK3CA (E542K) mutation in 30% of the cases. Kalsi *et al.* reported that E542K mutation in PIK3CA significantly alters the conformational behaviour of interacting residues, thus preventing the participation of these residues in protein-protein interaction. The regulatory control of p85α (PIK3R1) over p110α is lost on account of deprivation of interaction between p110α and p85α, prolonging the activity of catalytic p110α subunit. Prolonged p110α activity is accounted as one of the main reasons for uncontrolled cell division (Kalsi *et al.*, 2016).

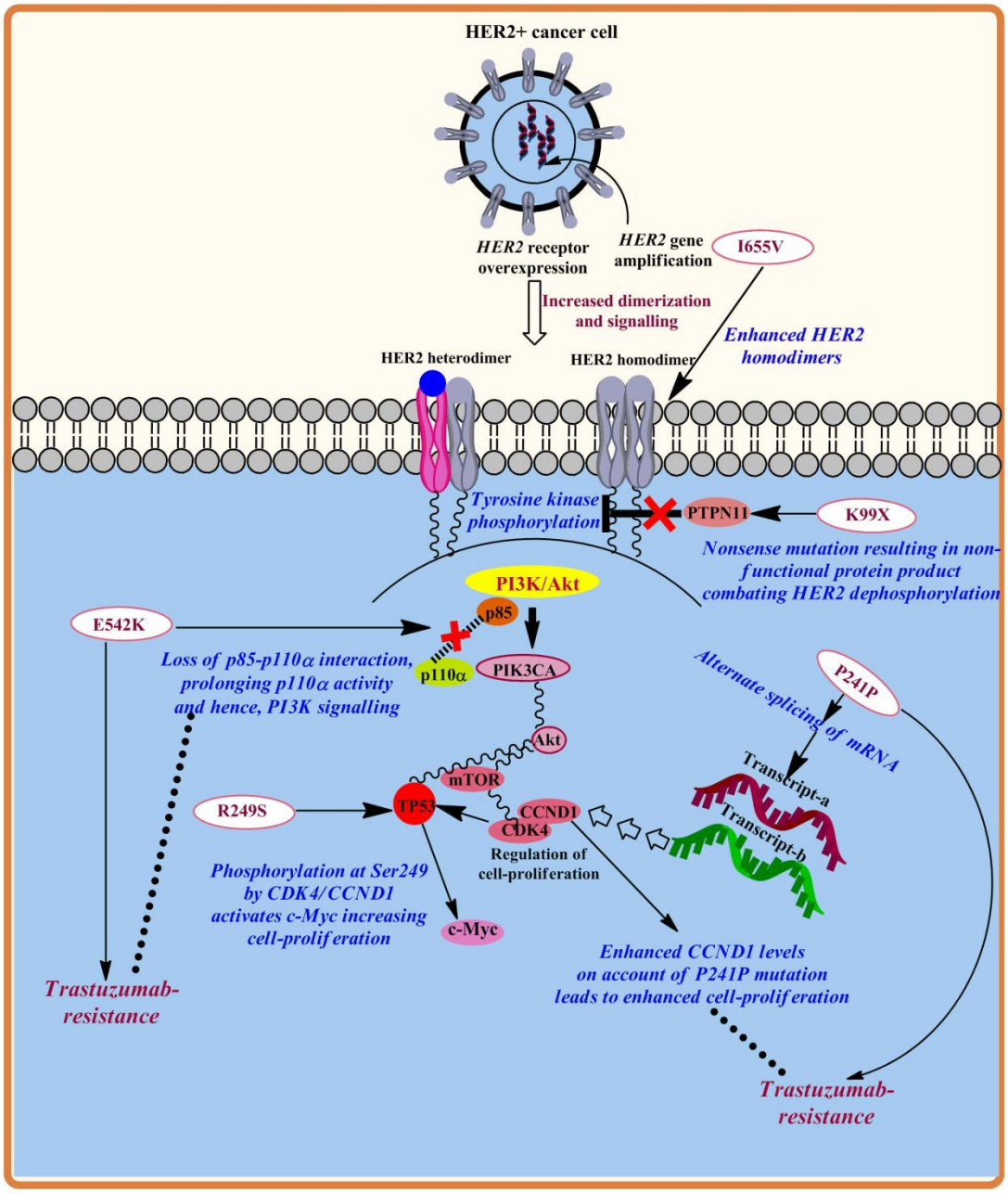


Fig. 35: Functional implications of genomic alterations in HER2 signal transduction. HER2 germline variant along with PTPN11 nonsense mutation exponentially multiplies HER2- mediated cell signaling. CCND1, PIK3CA and TP53 mutations lead to uncontrolled cell-division and hence, elevated tumourigenesis. Mutant CCND1 and PIK3CA confer resistance towards trastuzumab therapy

TP53 is a tumor-suppressor gene that has been found to be frequently mutated in HER2-positive breast cancers (Darb-Esfahani *et al.*, 2016). TP53 (R249S) was found to be mutated in 40% of the patients in our study. TP53 R249S is a hotspot and gain-of-function mutation promoting tumorigenic events. This TP53 mutation affects the amplification of cell-cycle regulatory proteins such

as including CCND1 and cyclin-dependent kinase 4 (CDK4). TP53 mutated at R249S, acts as a substrate of CDK4/CCND1 in the G1/S phase of cell-cycle. Liao *et al.* found that upon phosphorylation at Ser 249 by CDK4/CCND1, TP53 promotes cell-proliferation *via* c-Myc activation (Liao *et al.*, 2017).

A poor clinical outcome in response to trastuzumab-treatment has been reported in many studies and various genomic alterations have been found to play a significant role in conferring poor response in HER2-positive breast cancer patients treated with trastuzumab (Singla *et al.*, 2017). Mutated CCND1 and PIK3CA were found to be significantly associated with poor clinical outcome in HER2-positive breast cancer cohort treated with trastuzumab in the current study. Patients harbouring mutant CCND1 (P241P) exhibited reduced DFS as compared to the patients who carried wild-type CCND1 (P241P). CCND1 (P241P) is associated with CCND1 overexpression and the latter has been reported to be associated with poor clinical response in patients on trastuzumab therapy (Tanioka *et al.*, 2014).

PI3K pathway has been found to be frequently mutated in trastuzumab-resistant breast cancer (Chandarlapaty *et al.*, 2012). Baselga *et al.* reported the association of mutant PIK3CA with favourable outcome in breast cancer patients, who didn't receive trastuzumab treatment (Baselga *et al.*, 2011). Hanker and colleagues reported that mutated PIK3CA expedites HER2-driven mammary tumorigenesis (Hanker *et al.*, 2013). The oncogenic PIK3CA mutations activate the PI3K pathway and have been reported to be associated with the poor response of breast cancer patients to trastuzumab (Berns *et al.*, 2007; Kataoka *et al.*, 2009; Esteva *et al.*, 2010; Cizkova *et al.*, 2013; Loibl *et al.*, 2014). In conformity with previously published data, we found a significant association of mutant PIK3CA (E542K) with worse clinical outcome in HER2-positive breast cancer patients on trastuzumab treatment. Patients carrying PIK3CA (E542K) showed significantly decreased DFS in response to trastuzumab in the current study. E542K has been reported to be associated with enhanced PI3KCA kinase activity and thus, over-activation of PI3K pathway (Bachman *et al.*, 2004; Oda *et al.*, 2005; McIntyre *et al.*, 2013). The functional implications of the significant genomic alterations observed in the present study have been demonstrated in Fig. 35.

The main drawback of the study was the availability of a very less number of tissue samples. In the current study, we focused to delineate HER2-interacting genomic alterations from HER2-positive breast cancer and impact on clinical outcome. Our results suggest that germline HER2 (I655V) variant and tissue-specific mutations including PTPN11 (K99X), CCND1 (P241P), PIK3CA (E542K) and TP53 (R249S) are associated with HER2-positive breast cancer. In addition, mutant CCND1 and PI3KCA showed a significant association with reduced DFS in patients on trastuzumab therapy and the results of the present study need to be confirmed in a larger cohort. If confirmed, the patients might be advised to get screened for CCND1 and PIK3CA mutations before undergoing trastuzumab therapy.

5.3. Synthesis and characterization of target compounds

Anilinoquinazoline is an important scaffold. Many of the TKIs targeting HER2 receptor such as lapatinib, canertinib and neratinib belong to 4-anilinoquinazoline class of inhibitors. As a result of which this scaffold attracted the attention of medicinal chemists for developing HER2 inhibitors (Singla *et al.*, 2018). Based on previous studies (Singla *et al.*, 2018), we designed the derivatives of anilinoquinazolines. The substituted aldehyde was heated with anthranilamide in the presence of catalytic iodine and methanol as a solvent yielding quinazolinone **(5)** (Fig. 35). This is a one-pot iodine-mediated annulations that presents a facile approach to a variety of 1,3-diazaheterocyclic compounds including quinazolinones, benzimidazoles and cyclic amidines (Tian *et al.,* 2015). The oxidative cyclization of the reaction intermediate **(3)** was mediated by iodine, as explained in the detailed mechanism shown in Fig. 36.

Quinazolinone **(5)** was refluxed at a temperature of 110^0C in excess of $POCl_3$ in the presence of tertiary amine. An intermediate dichlorophosphate analogue **(6)** was formed giving 4-chloroquinazoline **(8)** at the end of reaction (Fig. 37) (Arnott *et al.,* 2011). The final step of synthesis of quinazoline-4-amine **(10)** from 4-chloroquinazoline **(8)** was a two-step process involving the formation of azide intermediate **(9)** (Fig. 38). **(8)** was first heated with sodium azide followed by subsequent addition of palladium-carbon catalyst and hydrazine hydrate, yielding **(10)** (Mohamed *et al.,* 2015).

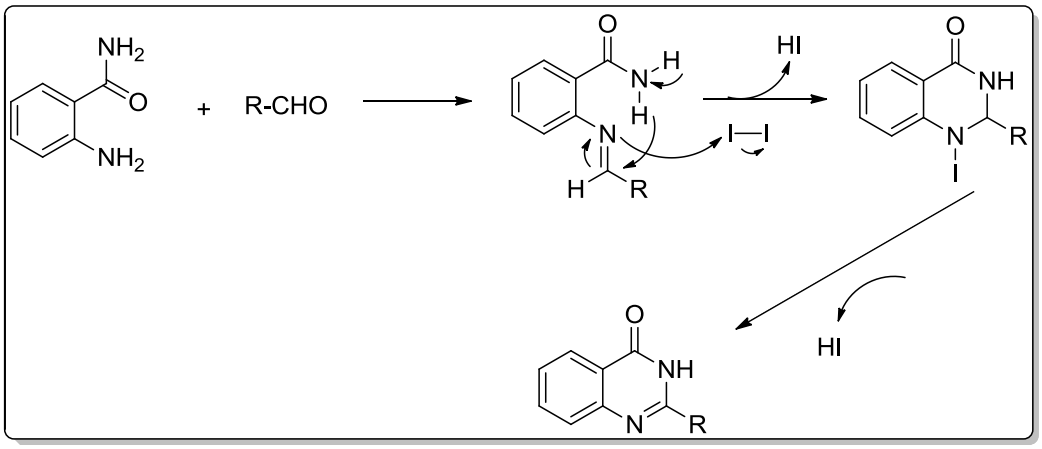


Fig. 36: Mechanism for the synthesis of quinazolinones

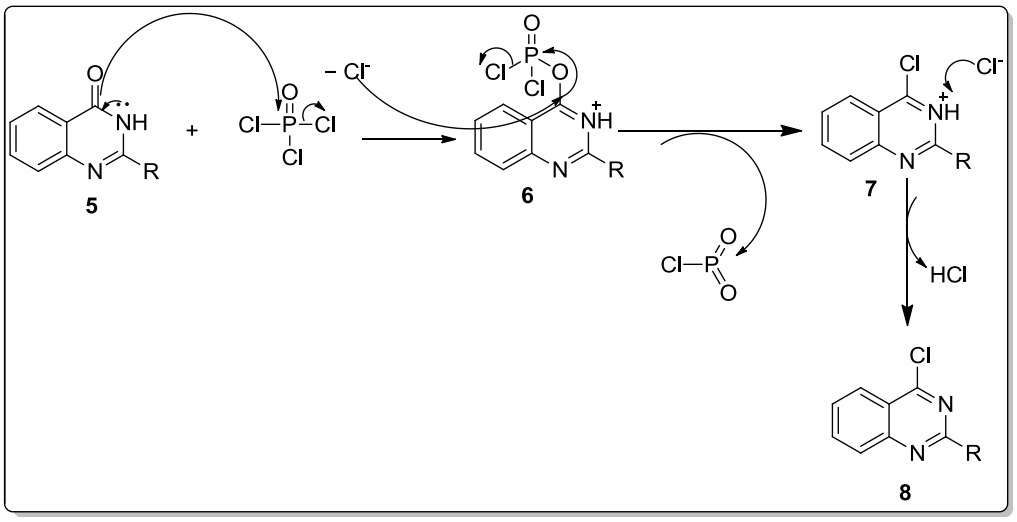


Fig. 37: Mechanism for the synthesis of 4-chloroquinazolinones

Further, a long alkyl chain in between the ring and –NH attached to the quinazoline ring was tried to incorporate. However, we failed to do so left with quinazoline-4-amine analogues. The purification of synthesized compounds was achieved via column chromatography and further, the characterization of all the compounds (**HS-2, HS-3, HS-5, HS-8** and **HS-9;** Fig. 39) was done by NMR and mass spectroscopy techniques.

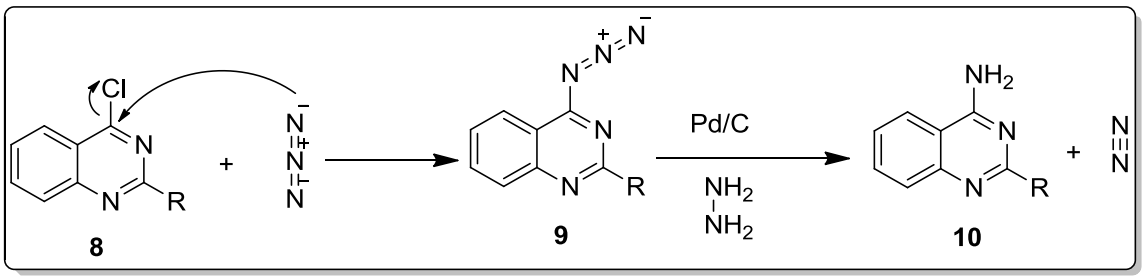


Fig. 38: Mechanism for the synthesis of quinazoline-4-amines

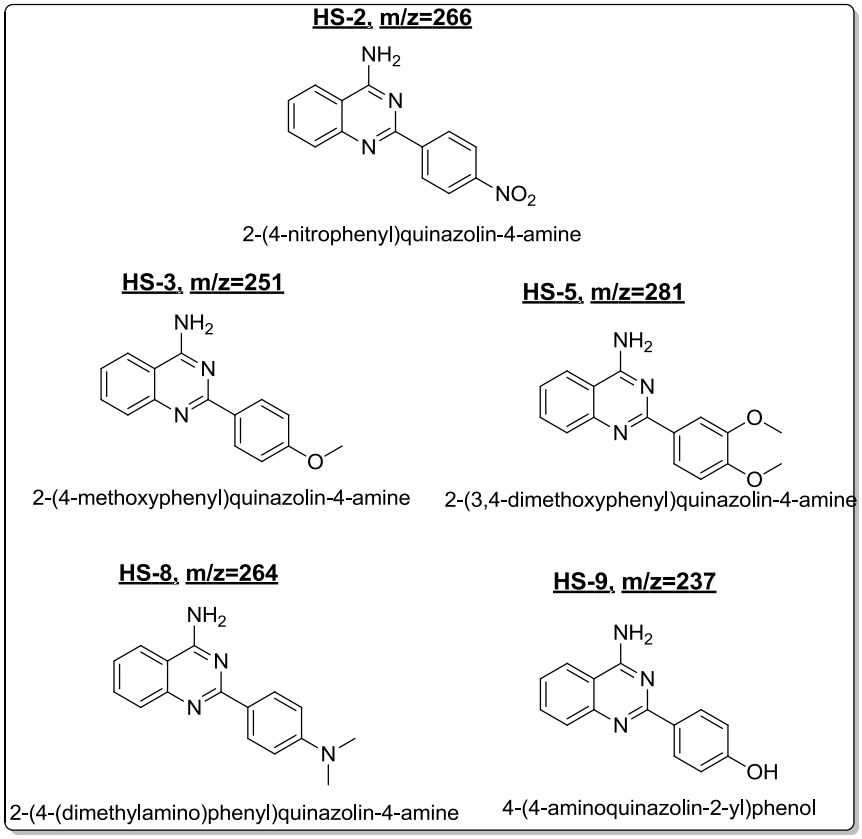


Fig. 39: Structures of the synthesized compounds

5.4. Biological evaluation of representative compounds

Three of the synthesized compounds (**HS-3, HS-5** and **HS-8**) were evaluated for their cytotoxicity profile against SKBR3 (HER2-positive breast cancer cells), MDA-MB-231 (triple-negative breast cancer cells) and FR-2 (normal human breast epithelial cells). Among the compounds evaluated for the anti-proliferative activity, the compound **HS-8** was the most potent that exhibited a higher inhibitory potential against SKBR3 (IC_{50}=2.8 µM) than MDA-MB-231 (IC_{50}=3.2 µM). This IC_{50} value is comparable with some previously reported anti-proliferative activities of HER2 inhibitors (Ding *et al,* 2017; Lin *et al.,* 2017). Amongst the synthesized compounds, **HS-8** didn't show any cytotoxicity against normal FR-2 cells. At the highest concentration of 5 µM, HS-8 inhibited more than 80% of SKBR3 cells, whereas HS-3 and HS-5 inhibited less than 45% of SKBR3 cells at the same concentration. However, both **HS-3** and **HS-5** showed <15% of cytotoxicity against normal FR-2 cell population. Some more derivatives need to be synthesized in order to develop SAR profile for the current series of compounds. Then, these analogues can be taken ahead to the next level of

biological evaluation of HER2 TK enzyme inhibitory activity. In addition, inhibition against a variety of other kinases can be evaluated to determine HER2 selectivity profile.

SUMMARY

HER2-positive breast cancer is an aggressive breast cancer subtype exhibiting HER2 overexpression and/or amplification. 20% of the breast cancer cases are HER2-positive as observed in the current study, as well. We aimed to understand the burden of pathogenic genomic alterations in HER2-positive breast cancer and their association with trastuzumab-response. To the best of our knowledge, this is the first study from India evaluating the association of genomic alterations with HER2-positive breast cancer as well as trastuzumab-resistance. There are various limitations associated with current targeted therapies for HER2-positive breast cancer, including the genomic alterations involved in development of trastuzumab-resistance. In order to address these issues, anilinoquinazoline-based inhibitors were designed, synthesized and biologically evaluated. The key findings of the study are:

- Postmenopausal HER2+ BC observed in 46% (46/100) of females and 8% (8/100) had a family history of cancer including breast and others. None of the patient characteristics associated significantly with HER2-positive breast cancer.
- A germline variant in the HER2 gene (I655V) associated significantly with HER2-positive breast cancer and with pesticide-exposure in patients. Hence, the individuals exposed to pesticides and bearing HER2 (I655V) variant are at a greater risk of developing HER2-positive breast cancer.
- Somatic mutations, including a PTPN11 nonsense mutation (K99X), a pathogenic CCND1 splice site variant (P241P), a hot spot missense mutation in PIK3CA (E542K) and a hot spot missense mutation in the TP53 gene (R249S) was detected 25%, 75%, 30% and 40% of the patients, respectively.
- HER2 (I655V), PTPN11 (K99X), CCND1 (P241P), PIK3CA (E542K) and TP53 (249) variants have emerged as important biomarkers in HER2-positive breast cancer risk, since these are involved in triggering HER2 homodimerisation, HER2 repression, abnormal cell-proliferation, enhanced PI3K signaling and c-Myc activation, respectively.

- In HER2-positive breast cancer patients treated with trastuzumab, a mutant CCND1 and/or PIK3CA gene was found to be associated with a poor clinical outcome (lower DFS). On the other hand, variation in HER2, PTPN11 and TP53 genes didn't show any significant association with clinical outcome.
- CCND1 (P241P) mutation results in CCND1 overexpression, further enhancing cell-proliferation and hence, conferring trastuzumab resistance.
- PIK3CA (E542K) mutation prolongs p110α activity by abolishing the p85-p110 interaction, leading ultimately to enhanced PI3K signaling and therefore, trastuzumab resistance.
- Five quinazoline-based compounds (HS-2, HS-3, HS-5, HS-8 and HS-9) were synthesized and of these, three (HS-3, HS-5 and HS-8) evaluated for anti-proliferative activity. **HS-8** was found to be most potent with IC_{50} value of 2.8 µM against HER2-positive breast cancer cells. The same compound exhibited slightly lower inhibitory activity against triple-negative breast cancer cells (IC_{50}=3.2 µM) and no cytotoxicity against normal breast epithelial cells.

FUTURE DIRECTIONS

- A larger cohort of HER2-positive breast cancer patients needs to be screened for genomic alterations in order to establish the results.
- Once established, the patients can be advised to get screened for genetic markers, particularly CCND1 and PIK3CA mutations so that HER2-targeted therapy can be tailored, accordingly.
- Some more compounds from the current series need to be synthesized to develop SAR.
- Kinase-inhibitory assays need to be performed against HER2 and other kinases in order to check the selectivity of these compounds.

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